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Corticotropin-releasing hormone regulation of immunoreactive beta-endorphin secretion in Leydig cells in the rat testis

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CORTICOTROPIN-RELEASING HORMONE REGULATION OF IMMUNOREACTIVE BETA-ENDORPHIN SECRETION IN LEYDIG CELLS IN THE RAT TESTIS

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ABSTRACT

CORTICOTROPIN-RELEASING HORMONE REGULATION OF IMMUNOREACTIVE BETA-ENDORPHIN SECRETION IN LEYDIG CELLS IN THE RAT TESTIS

by

Nahida Lucia Eskeland

Advisor: Beth S. Schachter, Ph.D.

Corticotropin-releasing hormone (CRH), a hypothalmic peptide, is the principal stimulator of pro-opiomelanocortin (POMC) synthesis and secretion in the pituitary gland. POMC and CRH syntheses have been documented in other tissues such as the testis, but a possible regulatory action of CRH on POMC in the testis was unknown. Therefore the current study evaluated various aspects of CRH action on POMC peptide secretion from testicular cells. CRH stimulated secretion of β-endorphin (βEP, a POMC peptide) from a subset population of Leydig cells in culture. The secretagogue acted in a dose-dependent fashion and was inhibited by a CRH competitive antagonist, demonstrating that POMC-producing Leydig cells have functional CRH receptors. Moreover, we have shown that CRH stimulated irβEP levels in testicular interstitial fluid in the intact testis of pubertal but not adult rats. These data suggest a physiological and developmental function for CRH in the testis.

Only short POMC-like transcripts that are missing the amino terminal signal-peptide

coding region have been reported in the rat testis. Using polymerase chain reaction, a full length pituitary-like POMC mRNA was detected in the rat testis, showing that the rat testis has a POMC transcript capable of encoding a prohormone that can go through processing, packaging and secretion pathway for regulated secretory peptides.

The effect of two physiologically relevant temperatures on the CRH/POMC system was analyzed since testicular function is known to be sensitive to thermal changes. At 34°C, CRH dose-response curves for both TM3 (a mouse Leydig cell-line) and AtT20 (a mouse pituitary cell-line) had significantly lower EC_{50}s than at 37°C. These studies suggest that CRH receptors or post receptor mechanisms may be sensitive to temperature changes.

Finally, possible source(s) of CRH synthesis in the rat testis was investigated. Using non-radioactive *in situ* hybridization on adult testis sections, CRH mRNA was detected predominantly in germ cells, suggesting that these cells may be a major source of CRH.

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FORMAT OF THESIS

This thesis is prepared according to the new guidelines of the City University of New York which permit the direct incorporation of published research articles as chapters. The thesis has an abstract, table of contents, general introduction, chapters, a general discussion, appendix and bibliography. Each chapter contains an abstract, a specific introduction, material and methods, results, discussion, tables and figures, and discussion. Copyright permission for the published chapter was obtained from the respective publisher.

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I. GENERAL INTRODUCTION

A. Paracrine regulation in the testis

Although the testis is primarily under the control of pituitary gonadotropins, there is increasing evidence that locally produced peptide hormones play important modulatory roles in the testis' response to the pituitary gonadotropins action on steroidogenesis and spermatogenesis. Sharpe (Sharpe, 1984) suggested that 1) the structural organization of the testis and 2) the organization and hormonal control of spermatogenesis are two factors that necessitate local control. The Sertoli, germ and interstitial cells each exert different effects on the other cell types, and these effects change according to the stage of the spermatogenic cycle. For example, seminiferous tubules located adjacent to Leydig cells modulate the morphology and function of these cells according to the stages of spermatogenic cycle in the tubules (Bergh, 1982; Syed et al., 1985a). In addition, co-culturing of isolated seminiferous tubules and Leydig cells affects Leydig cell testosterone secretion. Moreover, when depletion of germ cells occurs after experimental cryptorchidectomy (transplantation of a previously descended testis from scrotal sac to the abdominal cavity), Sertoli cell proteins such as androgen binding protein (ABP) and inhibin are drastically reduced (Jegou et al., 1983; Sharpe et al., 1986). When germ cells are selectively depleted (by X-irradiation), secretion of plasminogen activator protein from the Sertoli cells is inhibited.

Many peptide hormones, possibly of local origin, have demonstrated actions on steroidogenesis within the testis (Sharpe et al., 1987). For example, testicular inhibin (Bardin et al., 1990; Franchimont et al., 1989), luteinizing hormone-releasing hormone (LHRH) (Sharpe et al., 1981, 1983b), oxytocin, and vasopressin (Adashi et al., 1984) stimulate testosterone production, while CRH inhibited gonadotropin-stimulated testosterone production (Ulisse et al., 1989). In addition, local growth factors such as seminiferous growth factor (SGF), and insulin-like growth factor I (IGF-I) increase the rate of cellular proliferation and differentiation, and affect steroidogenesis (Bellve et al., 1989).

The evidence for local synthesis of regulatory peptides in the testis has come from a variety of studies that identify either the specific proteins, their corresponding mRNAs, or both. For example, the mRNAs encoding inhibin (Bardin et al., 1990), CRH (Thompson et al., 1987), oxytocin (Ivell et al., 1990), and POMC peptides (Chen et al., 1984; Pintar et al., 1984), is detected in testis, and peptide immunoreactivity is detected in cultured testicular cells (Fabbri et al., 1988, 1990; Morris et al., 1988). Moreover, the expression of a number of these peptides is regulated by the seminiferous tubules and/or other peptide hormones. For example, the expression of inhibin (Bhasin et al., 1989) and POMC peptides (Gizang-Ginsberg et al., 1985) in the testis depends on the stage of the seminiferous tubules. In addition, immunoreactive concentrations of CRH and βEP in testicular extracts or testicular interstitial fluid (TIF) are several fold higher than concentrations of these peptides in plasma and hypophysectomy does not completely abolish the

immunoreactivity of CRH and POMC-related peptides in the testis (Valenca et al., 1986; Yoon et al., 1988). These data suggest local paracrine and/or autocrine roles for these peptides. The site(s) of synthesis and action in the testis for the majority of the peptides mentioned above are not yet fully defined.

In the testis, POMC biosynthesis appears to occur in at least three distinct cell types. Leydig cells (Pintar et al., 1984), immature spermatocytes, and spermatogonia (Cheng et al., 1985). Imunoreactive CRH-like material (irCRH) has been shown in the testis, and specifically in Leydig and germ cells (Yoon et al., 1988; Audhya et al., 1989). Fabbri *et al.* (1990) demonstrated irCRH in media from Percoll-purified Leydig cell cultures, suggesting that these cells are capable of synthesizing CRH. It is not yet clear if Leydig cells are the primary source of CRH, or if other cells of the testis may be synthesizing the peptide.

Recently investigators examined the action of some of the local testicular factors on end-points other than steroidogonesis in Leydig cells. For example, Valenca demonstrated an acute stimulation of irβEP and immunoreactive adrenocorticotropin (irACTH) concentrations in TIF by the hypothalamic LHRH (Valenca et al., 1986). There is evidence that Sertoli cells in the rat produce an LHRH-like peptide (Sharpe et al., 1981, 1987).

B. Brief background on POMC and CRH

The pituitary gland is the major site of synthesis of POMC (Antoni, 1986; Lundblad et al., 1988), the precursor of several different endocrine hormones such as

adrenocorticotropin (ACTH), the opioid βEP and melanocyte-stimulating hormone (MSH) (Eipper et al., 1980). The hypothalamic peptide CRH is the principal stimulator of POMC peptide secretion from the pituitary in response to stress (De Souza et al., 1985; Lundblad et al., 1988; Dunn et al., 1989). CRH regulates pituitary POMC peptide release via an interaction with a specific cell membrane receptor on pituitary corticotrophs (Wynn et al., 1983).

CRH messenger RNA (CRH mRNA) (diagram of the CRH gene is shown in Appendix I) is found in a wide variety of extrahypothalamic tissues including olfactory bulb (Imaki et al., 1989), pituitary, brain, spinal cord, adrenal, testis (Thompson et al., 1987), and placenta (Grino et al., 1987). POMC mRNA is also found in many tissues including adrenal, thymus and testis (Lacaze-Masmonteil et al., 1987) suggesting that these tissues have the capacity to synthesize CRH and POMC. In addition, CRH and POMC peptide hormones are detected in the placenta (Margioris et al., 1988) and adrenal (Evans et al., 1983), and CRH can stimulate POMC peptide secretion from the placenta (Petraglia et al., 1987a; Margioris et al., 1988). Also, CRH binding sites have been demonstrated in testis, spleen, liver, kidney and adrenal (Jitendra et al., 1985; Webster et al., 1988). Hence, it may be that many tissues have local (paracrine or autocrine) CRH/POMC interaction that are similar to that in the hypothalamic/pituitary axis.

C. <u>POMC messenger ribonucleic acid (mRNA) in the testis</u>

In the early 1980s, Pintar *et al.* (1984) and Chen *et al.* (1984) characterized the

POMC mRNA (diagram for the POMC gene is found in appendix II) (Jeannotte et al., 1987) in the adult rat testis by Northern blot analysis. Pintar and coworkers (1984) and Gizang-Ginzberg and coworkers (1985) localized the POMC mRNA in rat and mouse Leydig cells using the *in situ* hybridization method. This POMC mRNA was at least 200nt shorter than the POMC mRNA of 1100nt in the pituitary. The testicular POMC mRNA has most of Exon3, which contains most of the protein coding sequence, but is missing Exons 1 and 2, and therefore lacks the codons for amino terminal signal peptide (Jeannotte et al., 1987). This POMC mRNA is approximately 100 times less abundant than the POMC mRNA in the pituitary (reviewed in Bardin et al., 1987). Other nonpituitary tissues also contain this short POMC mRNA, such as duodenum, kidney, colon, liver, and spleen (DeBold et al., 1988).

In pituitary corticotrophs, the regulated POMC is packaged into secretory vesicles and undergoes cleavage before the POMC-derived products are secreted (Glembotski, 1981; Gumbiner et al., 1981, 1982; Tooze et al., 1986, 1987). Recently, Clark *et al.* (Clark et al., 1990), in their transfection studies on GH_3 cells, demonstrated that cells containing the full-length POMC transcript were able to secrete βEP and ACTH hormone (another POMC-derived peptide). Cells transfected with an expression vector for the short transcript were unable to secrete any of these peptides. These data suggest that the pituitary-like POMC message may be necessary in successful packaging and secretion of the POMC-derived peptides.

The lack of detectable signal-peptide coding sequences in the POMC-like RNA in

tissues such as the testis raised questions about the source and fate of POMC peptides in this tissue. Are POMC peptides synthesized from the short transcript and therefore, because they lack the signal peptide, not secreted or constitutively secreted? Or, is there regulated secretion of POMC peptides made from a low abundance, full-length POMC mRNA that encodes POMC prohormones that is precursor to regulated secretory products? Finding answers to these questions formed part of the basis for the studies outlined in this dissertation.

During the course of our research, Lacaze-Masmonteil and coworkers (1987), using S_1 nuclease assay (a sensitive method to detect low abundant mRNA), were able to identify, in the human testis, a long POMC mRNA similar in length to the pituitary POMC mRNA, but in levels approximately 100 times less than the short transcripts in the adult human testis.

D. POMC-derived peptide and CRH function in the testis

Biochemical and physiological responses to several POMC peptides have been documented to occur in the testis (; Orth et al., 1986; Boitaini et al., 1986; Bardin et al., 1987; Knotts et al., 1988). Opiate receptors are found on Sertoli cells in culture (Fabbri et al., 1985) and βEP inhibits follicle-stimulating hormone (FSH)-stimulated androgen binding protein (ABP) production from these cells. Intratesticular injection of the opiate antagonists nalmefene or naloxone increased the compensatory hypertrophy following unilateral castration (Bardin et al., 1987; Cicero et al., 1989). The antagonists also increased rat androgen binding protein suggesting that βEP

and/or other opioids (enkephalins and dynorphins which are also localized in the testis) (Kilpatrick et al., 1986; Douglas et al., 1987; Garrett et al., 1989) inhibit Sertoli cell growth and secretion during early testicular development. βEP acutely stimulated testosterone secretion in Leydig cell cultures prepared from 20-day-old rats (Bardin et al., 1987). In contrast, perfusion of βEP or naloxone in intact adult rat testes *in vitro* or *in vivo* had no effect on testosterone levels while ACTH increased testosterone secretion (Sharpe et al., 1987; Juniewicz et al., 1988). α-MSH, another POMC peptide, stimulates the production of ABP and Sertoli cell number (Boitaini et al., 1986; Orth, 1986). ABP and Sertoli cells are essential for normal spermatogenesis.

From these studies has come the suggestion that POMC peptides which are synthesized in the testis may have paracrine and/or autocrine functions in the male gonads. Implicit in this suggestion is the assumption that POMC peptides are actively secreted in a regulated fashion from the testicular cells in which they are made. Recently, Fabbri *et al.* (1988) demonstrated gonadotropin stimulation of irβEP secretion from cultures of fetal Leydig cells; in addition, Valenca and coworkers showed LHRH and LH stimulation of irβEP secretion from the whole adult rat testis (Valenca et al., 1986). It was also shown that CRH inhibited luteinizing hormone(LH)-stimulated testosterone secretion from Leydig cells in culture (Ulisse et al., 1989). From these results, Fabbri, in his review article on opioids (Fabbri, 1990), suggested an antireproductive role for βEP and CRH in the testis, in addition to their known antireproductive functions in the brain and the hypothalamus

(Rivier,C et al., 1984, 1986; Petraglia et al., 1987b; Barbarino et al., 1989a,b; Fabbri et al., 1989).

E. Developmental regulation in the testis

Testosterone production and spermatogenesis are essential for normal male reproduction. Their onset are the markers for puberty. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are the major regulators of these markers. In prepubertal animals, LH and FSH, which are in low circulating levels, do not affect testicular maturation (Purvis et al., 1979; Pilsworth et al., 1981; Ritzen et al., 1981; Russel et al., 1987). At puberty, the hypothalamic LHRH stimulates the secretion of LH and FSH from gonadotrophs in the pituitary and in turn, these peptides, in high circulating levels, stimulate testosterone production and spermatogenesis. In addition, the maturation process involves changes in expression of genes for a variety of secretory peptides, few of which are under gonadotropin control. For example, inhibin and POMC expression is highest at puberty (Shaha et al., 1984; Chen et al., 1987; Rivier,C et al., 1988; Yoon et al., 1988; Keeping et al., 1990), and are stimulated by LH or FSH. In the mouse, irβEP staining in interstitial cells progressively increases throughout fetal life; at the time of birth, the percentage of irβEP stained cells was found to be 55% of interstitial cells. The number declines after birth to 12% at 5 days of age, increases to almost 100% by 40 days and after. IrβEP and irACTH declines after hypophysectomy and increases after human chorionotropic hormone (hCG) treatment. In the rat, Yoon *et al.* (1988)

demonstrated that irCRH level was high at 10 days of age, decreased sharply at 20 days, and rebounded at 60 and 90 days. Hypophysectomy decreased irCRH levels in the testis. In addition, changes in LH, FSH and vasopressin receptor number and steroidogenesis during development have been observed (Huhtaniemi et al., 1981, 1982; Chase et al., 1983; Tahri-Joutei et al., 1989b). These changes in the expression or action of local peptides at different ages of the animal led us to investigate whether the action of CRH in the testis is developmentally regulated.

F. Temperature changes and chryptorchidism

The testes of many mammalian species reside in the scrotum, which serves a thermoregulatory function and maintains an optimum environment for spermatogenesis. The normal scrotal temperature is about 32-34°C which is several degrees cooler than the core body temperature of 37°C. Evidence suggested that the cooler environment of the scrotum seems to be essential for maintenance of normal testicular structure and function in the adult animal. For example, profound tubular damage (Chowdhury et al., 1964, 1970; Davis et al., 1966), and morphological and functional changes in Sertoli and Leydig cells have been extensively documented in artificial cryptorchidism (Risbridger et al., 1981; Jegou et al., 1983; Huhtaniemi et al., 1984; Lunstra et al., 1988; Sirvent et al., 1989). Decrease in testicular weight was observed as well as hyperresponsive testosterone response to hCG stimulation in vitro (Iturriza et al., 1979), decrease in arginine vasopressin (AVP) (Tahri-Joutei et al., 1989a), LH, prolactin (PRL) and FSH receptors occurred in the adult rat (Risbridger

et al., 1981; Huhtaniemi et al., 1984; Bergh et al., 1987) as well as decrease in DNA synthesis and alteration in several steroidogenic enzymes' activities (Damber et al., 1983; Fujisawa et al., 1988; Hotta et al., 1988; Nakamura et al., 1988). Given the sensitivity of the organ to small temperature changes, one wonders how such changes influence cell-cell interaction through the expression and the action of local paracrine regulators. Intratesticular irCRH has been shown to decrease when rat testis was moved to the abdomen (Yoon et al., 1988). This is not surprising since irCRH is detected in germ cells of the tubules and germ cells are very vulnerable to temperature changes.

It has also been reported that levels of irβEP and irACTH in the blood are elevated during hyperthermia or hypothermia (Nakane et al., 1985; Vescovi et al., 1990). It is not known if these increases are at the brain or pituitary levels. These documentations lead us to investigate the role of small temperature changes, as a stress stimulus, on the action of CRH on POMC peptides.

G. Specific aims

1) to look at the effect of CRH on irβEP secretion from Leydig cells *in vitro*. The reverse hemolytic plaque assay (RHPA) will be used to detect irβEP secretion from individual cells. Leydig cells will be identified by immunocytochemistry using specific monoclonal antibodies to Leydig cell surface antigens.

2) to assess the physiological role of CRH on testicular βEP in the whole animal. This study will help determine whether CRH is a local regulator in the testis. Since the stages of spermatogenesis seem to have influence on the behavior of testicular cells, pubertal and adult animals will be used to determine whether the action of CRH is modulated by the testicular microenvironment.

3) to establish whether the rat testis has a full length pituitary-like POMC mRNA capable of synthesizing a POMC protein whose secretion is regulated.

4) to study the effect of temperature on the CRH/POMC system since it is known that spermatogenesis, and to a lesser degree, steroidogenesis are sensitive to small temperature changes. In addition, CRH/POMC system is known to be responsive to stress stimuli.

5) to localize the site(s) of CRH synthesis in the testis.

II. STIMULATION OF IRβ-ENDORPHIN SECRETION BY CORTICOTROPIN-RELEASING HORMONE IN PRIMARY RAT LEYDIG CELL CULTURES*.

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1. ABSTRACT

CRH, a hypothalamic peptide, is a potent stimulator of POMC synthesis and secretion in corticotropic cells of the pituitary. POMC peptides have also been localized in other cells such as testicular Leydig cells. Drawing on these observation, a reverse hemolytic plaque assay and immunocytochemistry were used to study the effect of CRH on secretion of the POMC peptide, βEP, from normal rat primary Leydig cell cultures. Our results showed that in enriched Leydig cell preparation treated with βEP antiserum (diluted 1:50) and complement (diluted 1:25), approximately 18% of the Leydig cells formed plaques. The addition of an increasing concentration of CRH (from 10^{-11}M to 10^{-7}M) increased the percentage of plaques by 83% and the average size of the plaque by 86%. When the CRH antagonist, CRH9-41 (10^{-6}M) was added in the presence of CRH, the increase in plaque number and average size was abolished. Preabsorption of the antiserum with βEP (2 μg/μl of antiserum) overnight at 4°C abolished the formation of plaques. These results demonstrate that the Leydig cells have CRH receptors and that irβEP secretion from these cells is stimulated by CRH.

2. INTRODUCTION

The pituitary gland is the major site of synthesis of POMC, the precursor of several different endocrine hormones. The hypothalamic peptide CRH is the principal stimulator of POMC peptide secretion from the pituitary. CRH regulates pituitary POMC peptide release via an interaction with a specific cell membrane receptor on pituitary corticotrophs (Lundblad et al., 1988).

Small quantitiies of POMC are produced in a wide variety of tissues throughout the body, including the testis, ovary, placenta (Bardin et al., 1987), and adrenal (Evans et al., 1983). In some organs more than one cell type may synthesize POMC. For example in the testis, the prohomrone is made in both Leydig cells and immature spermatocytes (Chen et al., 1984; Pintar et al., 1984; Cheng et al., 1985; Gizang-Ginsberg et al., 1985).

Recent studies have shown that immunoreactive CRH (Yoon et al., 1988) and CRH mRNA (Thompson et al., 1987) are present in the testis, suggesting that some cells in this tissue have the capacity to synthesize CRH. The peptide is also detected in placental perfusates (Margioris et al., 1988), and in this tissue it can stimulate POMC peptide secretion (Petraglia et al., 1987b; Margioris et al., 1988). CRH-binding sites have also been demonstrated in testis, spleen, liver, kidney, and adrenal (Jitendra et al., 1985; Webster et al., 1988). Hence, it may be that many tissues have local CRH/POMC stimulus/ response systems which, at least qualitatively, resemble that

in the hypothalamic/pituitary axis.

The current study was designed to test the effect of CRH on the release of the POMC-derived peptide βEP from testicular Leydig cells. We chose to use the RHPA in our studies instead of RIA because RIA does not discriminate between βEP-secreting and nonsecreting cells. Moreover, the RHPA, but not the RIA, allows for subsequent immunocharacterization of the specific cells that secrete βEP. Finally, other workers have reported a lack of success in detecting secreted βEP in adult Leydig cell cultures by RIA, possibly due to a high level of secreted proteases in their cultures (Fabbri et al., 1988) (Bardin, W., personal communication).

The data in the current study provide the first direct evidence that Leydig cells of the adult rat actively secrete irβEP and that this action is specifically stimulated by CRH. Hence, the results demonstrate that Leydig cells have functional CRH receptors, and that CRH may have a paracrine or autocrine function in the testis.

3. MATERIALS AND METHODS

3.1 *Chemicals*

CRH, α-helical CRH-(9-41) (a CRH competitive antagonist), and human βEP were obtained from Peninsula Laboratories (Belmont, CA); guinea pig complement, penicillin-streptomycin, sterile saline, HEPES-buffered solution and Dulbeco's Modified Eagle's Medium (DMEM) were obtained from Gibco (Grand Island, NY); Staphylococcal protein-A, poly-l-lysine, BSA, chromium chloride hexahydrate, paraformaldehyde were from purchased from Sigma (St. Louis, MO); sheep blood preserved in modified Alsever's solution was obtained from Rockland (Gilbertsville, PA); collagenase was obtained from Millipore Corp. (Freehold, NJ); and Percoll was from Pharmacia (Uppsala, Sweden). Reagents for avidin-biotin peroxidase immunocytochemistry were obtained from Vector Laboratories (Burlingame, CA), and 3,3'-diaminobenzidinetetrahydrochloride was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI).

3.2 *Antisera*

The βEP antiserum (EP-3) was the generous gift of A. Liotta (NIH). This antiserum, which was raised against synthetic porcine βEP, has been extensively characterized (Liotta et al., 1981); in particular, it cross reacts on an equimolar basis

with camel βEP, camel βEP-(1-27), the human forms of these peptides, their α-N-acetyl derivatives, and the common precursor molecules. It does not react with α- or γ-EP.

The Leydig cell specific monoclonal antisera LC-1C6 and LC-6H6 were gifts of E.M. Eddy (Research Triangle Park, NC). In enzyme-linked immunosorbent assays on isolated testicular cells and in immunocytochemical studies on fixed testis sections, these antisera reacted only with Leydig cells (Hedger et al., 1986). Approximately 60-70% of the isolated cells stained with the monoclonal antibodies compared to 83.1% that gave positive staining for 3β-hyddroxysteroid dehydorgenase (Hedger et al., 1986, 1987). In our Leydig cell preparations a similar percentage of cells stained with the monoclonal antisera, as described below.

3.3 *Animals, cell dispersion, and purification*

Adult male Sprague-Dawley rats (275-300 gm) were obtained from Charles River Laboratories (Wilmington, MA). Rats were housed under standard laboratory conditions, with free access to food and water. Animals were killed by CO_2 asphyxiation. Rat testes were perfused through the testicular artery with saline, using a 28.5 gauge needle, to remove most of the red blood cells. The testes were decapsulated and enzymatically dispersed with collagenase, and Leydig cells were purified by the method of Anakwe et al. (Anakwe et al., 1985) with few modifications. For each assay, four testes were placed in a 50-ml conical tube with 6 ml collagenase solution (1.2 mg/ml collagenase in DMEM-0.1% BSA containing 25

mM HEPES solution, 10 units penicillin and 10 μg streptomycin) and incubated for 20 min at 34°C in a shaking water bath at 80 cycles/min. The cell suspension was diluted with 4 vol DMEM-0.1% BSA solution and kept at room temperature for 2 min to allow the seminiferous tubules to settle. The supernatant was decanted through 4 layers of gauze into a fresh 50 ml tube. The tubules were washed once with DMEM-0.1% BSA solution, and the resulting supernatant collected. The combined supernatants were centrifuged twice at 118 x g for 5 min, and the pellet containing the interstitial cells was furthur purified using Percoll density gradient centrifugation. Briefly, 20mls of an isotonic 80% Percoll solution were layered under 20 ml of an isotonic 40% Percoll solution in a 50-ml conical tube. Interstitial cells were layered on top of the gradient in 3 ml DMEM-0.1% BSA solution and centrifuged for 20 min at 500 x g. The Leydig cell fraction formed a discrete band at the interface of the two Percoll fractions. At least 60% of the cells in this band stained with the monoclonal antibodies LC-1C6 and LC-6H6 to Leydig cell surface antigens (Hedger et al., 1986). Viability of the cells was more than 90% by trypan blue exclusion. This fraction was collected by aspiration and diluted with 3 vol DMEM-0.1% BSA solution, and the cells were collected by centrifugation, washed twice with DMEM-0.1% BSA solution, and suspended in the solution for the assay.

3.4 *RHPA*

The RHPA was performed as described by Smith and coworkers (Smith et al., 1986) (see diagram in appendix III). Two chambers per slide were constructed. The

cell mixture containing equal volumes of enriched rat Leydig cell preparations (approximately $2x10^5$ cells/ml) and an 18% (vol/vol) suspension of protein-A-coated ovine erythrocytes was infused into each chamber and incubated in a humidified CO_2 incubator for 45 min. Unattached cells were removed by infusing DMEM-0.1% BSA solution. The antiserum βEP (diluted 1:50) and the hormone CRH in DMEM-0.1% BSA solution were added, and slides were incubated at 37 C for the indicated length of time. Then, complement (diluted 1:25) was added, and the slides were furthur incubated for 1 h. The reaction was stopped by immersing the slides in 2% paraformaldehyde in phosphate buffered saline (PBS). The fixation continued overnight, then slides were rinsed, stained with toluidine blue or cresyl violet, and stored in 0.1 M phosphate buffer at 4°C.

3.5 *Immunocytochemistry*

In some experiments the slides from the RHPA were processed for avidin-biotin-peroxidase immunocytochemistry. Briefly, coverslips were removed, and slides air dried, then dessicated for 30 min to insure that cells remained attached. Slides were then soaked in 0.15 M PBS and incubated in 1) diluted normal goat serum for 20 min to block nonspecific binding; 2) primary antiserum (mixture of LC-1C6 and LC-6H6, each at a 1:20 dilution) overnight at 4 C, followed by washing; 3) biotinylated goat anti-mouse IgM (diluted 1:222) for 45 min and washed; and 4) avidin DH and biotin-conjugated peroxidase H for 45 min and washed. The reaction product was developed in freshly prepared DAB and 0.003% hydrogen peroxide in

0.04 M phosphate buffer for 5 min. After thorough rinsing, the slides were counterstained as described above, dehydrated and coverslipped.

For negative controls, primary antiserum was either omitted or replaced by normal mouse ascites fluid. In addition, an enriched Leydig cell preparation was divided in half, and one half was exposed to βEP antiserum for 2 h before immunocytochemistry, while the other was tested with the monoclonal antibodies directly. No difference in the percentage of cells staining with the monoclonal antibodies was observed in the two samples, verifying that the βEP antiserum did not interfere with immunocytochemical detection of Leydig cells. Immunocytochemistry caused some random loss of red blood cells from the chambers, but did not change the measured plaque areas. Moreover, while ghost cells were visible around plaques befor immunocytochemistry, most were lost after this procedure.

3.6 *Quantitative analysis*

The analysis was done using Axiomat Image Analyzer System, and the plaque areas were calculated using the Drexel analysis program (Feingold et al., 1986). In all experiments, chambers without antagonist were tested in duplicate, while each condition with antagonist was tested in a single chamber. Areas of 30-60 plaques were measured for each test and then averaged. The percentage of cells with plaques was determined after screening at least 150 cells/condition. Mininmum plaque diameter corresponded to 2 red blood cell diameters. Analysis of variance was used to determine overall differences among the different experimental

conditions.

4. RESULTS

4.1 The rate of plaque formation on irβEP-producing cells in the presence or absence of CRH

In prelimenary studies secretion of irβEP from Percoll gradient-enriched Leydig cell preparations was examined in the presence or absence of CRH (Figs. 1 and 2). Samples were incubated with βEP antiserm with or without CRH for varying times, then complement was added. As seen in Figure 1, samples cultured in the absence of CRH showed a time-dependent increase in the percentage of plaque-forming cells, reaching about 17% at 4 h. Samples incubated with 10^{-7} M CRH showed an increase in the rate of plaque formation, reaching an apparent plateau (also of ~17% of the cells) at 2 h. As seen in Figure 2, the mean plaque area continued to increase during the 4-h period, and the mean plaque size at all time ponts was greater in the presence than in the absence of CRH. No plaques were seen in chambers lacking βEP antisera or complement or in samples tested with antiserum preincubated overnight at 4 C with 2 μg synthetic human βEP/μl antiserm. These results show that βEP secretion from individual cells could be readily detected by this assay, and that the cells remain viable and capable of βEP secretion for several hours. Importantly, these results suggest that βEP secretion is stimulated by CRH. Therefore, a more thorough analysis of the effect of CRH was conducted.

4.2 Monoclonal antibodies specific to Leydig cell surface antigens

Figure 1. The rate of plaque formation by irβEP-producing cells in the presence or absence of CRH. The analysis was made by counting at least 200 cells/chamber in duplicate chambers for each timepoint. Values shown are the mean ± SD.

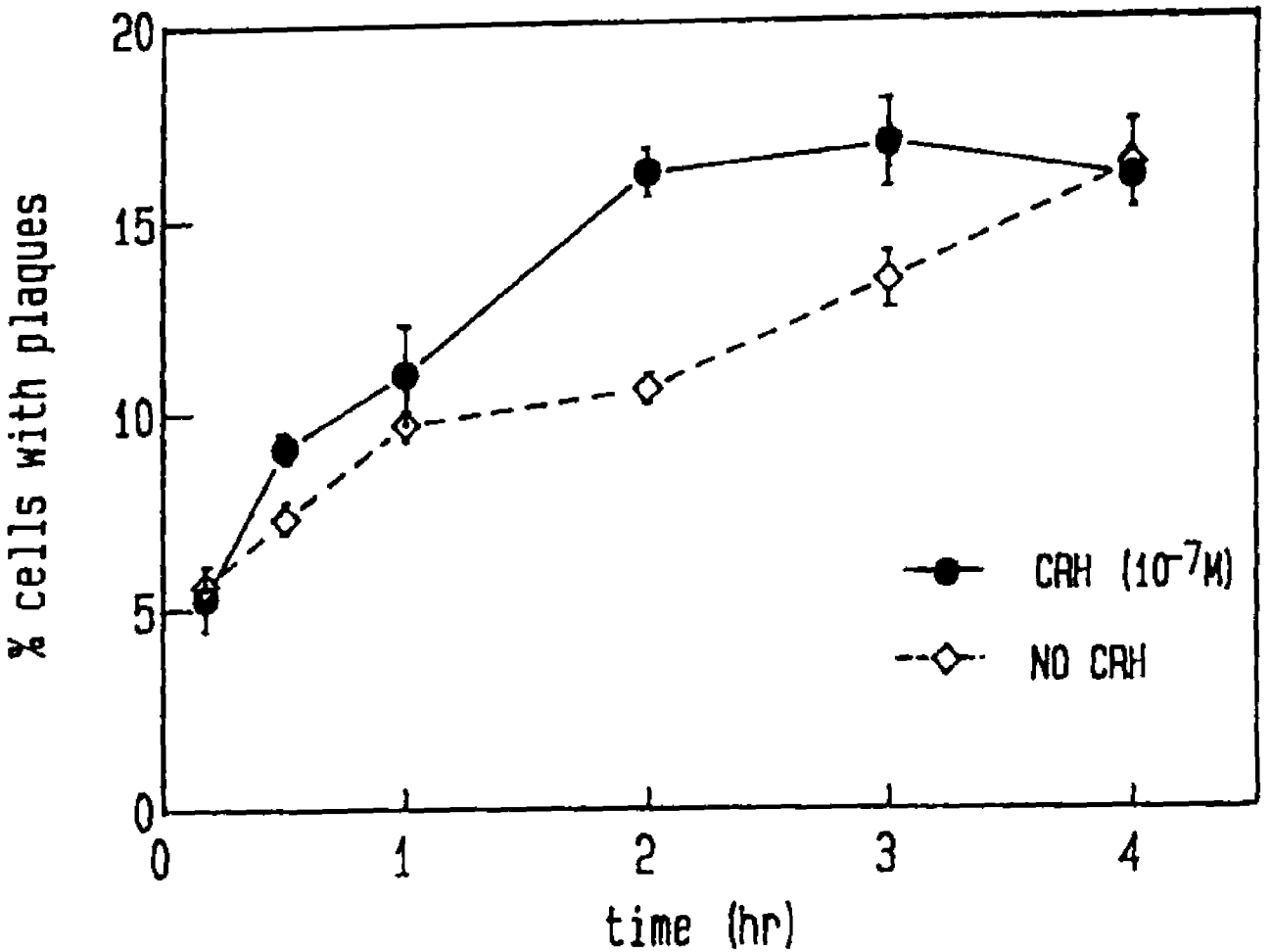
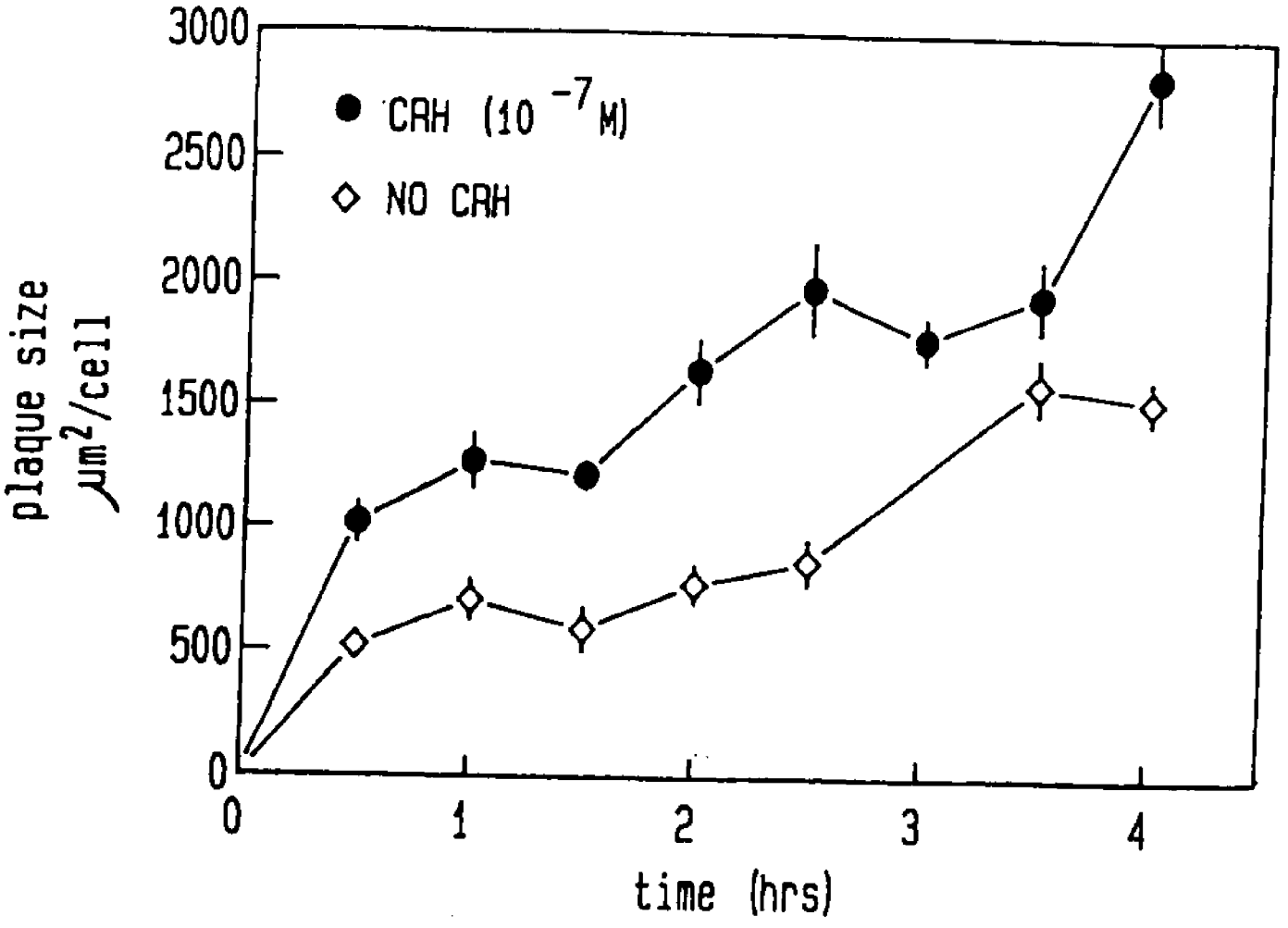

20
15
10
5
0
% cells with plaques
0
1
2
3
4
time (hr)
CRH (10^{-7}M)
NO CRH

Figure 2. The rate of change in irβEP plaque size in the presence or absence of CRH (10^{-7} M). The values are the mean ± SE of determinations made from 25-30 plaques in a chamber.

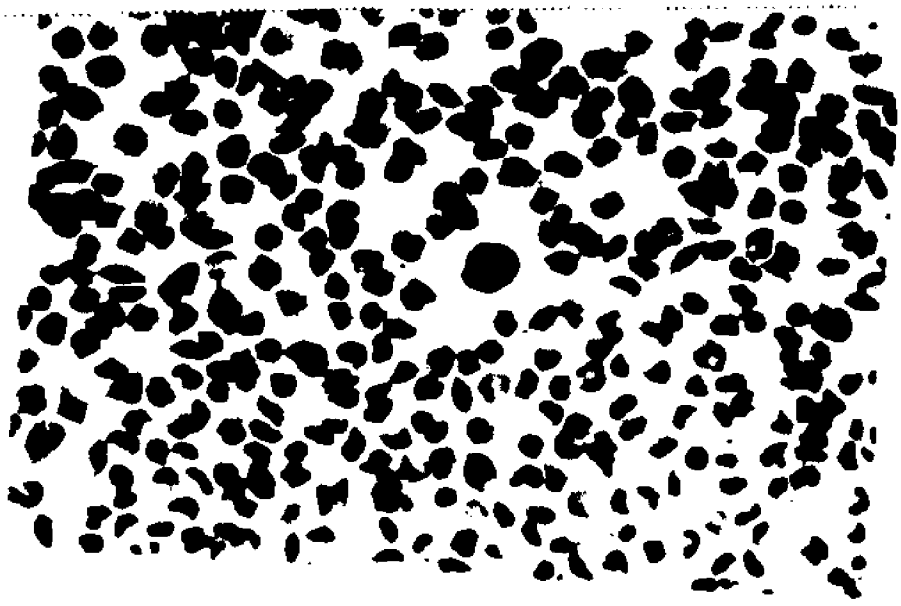

3000
2500
2000
1500
1000
500
0
0
1
2
3
4
plaque size
μm²/cell
time (hrs)
CRH (10⁻⁷ M)
NO CRH

To restrict our investigation to Leydig cells, in some assays we assessed only those cells that could be stained by a combination of Leydig cell-specific monoclonal antisera. Therefore, after the RHPA, cells were analyzed immunocytochemically as described in Material and Methods section. Approximately 60% of the cells stained with the monoclonal antibodies. Of the unstained cells (40%), only 4.5% had plaques in the presence of CRH at 10^{-7} M. Examples of cells that formed plaques due to irβEP secretion and were subsequently stained with Leydig cell antibodies are shown in Figure 3. Figure 3A shows a small plaque formed around a Leydig cell cultured in the absence of CRH; Figure 3B shows a larger plaque formed around a cell maintained in the presence of 10^{-8}M CRH.

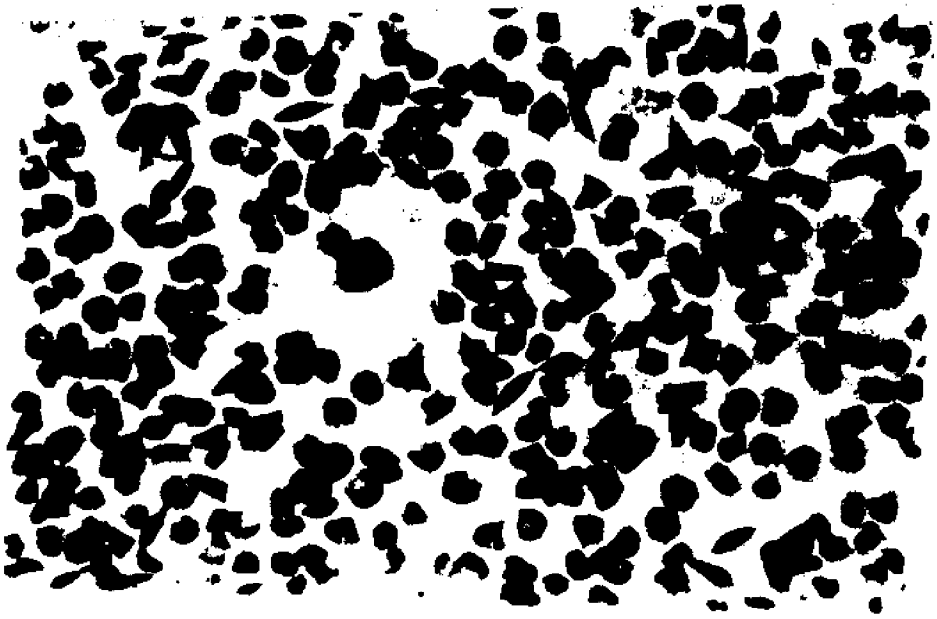
4.3 CRH dose-response curves in the presence or absence of CRH-(9-41)

A complete CRH dose response curve was then obtained on samples incubated for 2 h. In addition, a parallel analysis was carried out in the presence of the CRH antagonist α-helical CRH-(9-41)(10^{-6} M). Figure 4 shows that a significant CRH concentration-dependent increase in mean plaque area occurs surrounding immunostained Leydig cells ($p < 0.05$), reaching an approximately 85% increase in the presence of 10^{-8}M CRH or more. In contrast, cells cultured in the presence of the antagonist showed no CRH-related increase in mean plaque size, suggesting that CRH stimulates irβEP through a classical membrane receptor dependent mechanism. In addition, it should be noted that small plaques were detected in the absence of added CRH, and there was no further reduction in the mean area of these plaques

Figure 3. IrβEP plaque-forming cells immunostained with Leydig cell-specific monoclonal antibodies. A, a cell tested under basal conditions; B, a cell tested after stimulation with 10^{-8} M CRH. Magnification, x320.



A



B

Figure 4. Dose-response curve of CRH effects on mean plaque size, which is an index of the amount of irβEP secreted from a Leydig cell. The RHPA was performed for 2 h. The mean size of the plaques in the antagonist [CRH-(9-41)] treated cultures remains at basal levels. Values in the top curve represent the mean ± SE of determinations made from 40-60 plaques in duplicate chambers. Values in the lower curve (+ antagonist)represent the mean ± SE of determinations made from 20-35 plaques in a chamber. The responses to CRH and the CRH antagonist in this experiment are representative of at least three separate RHPAs.

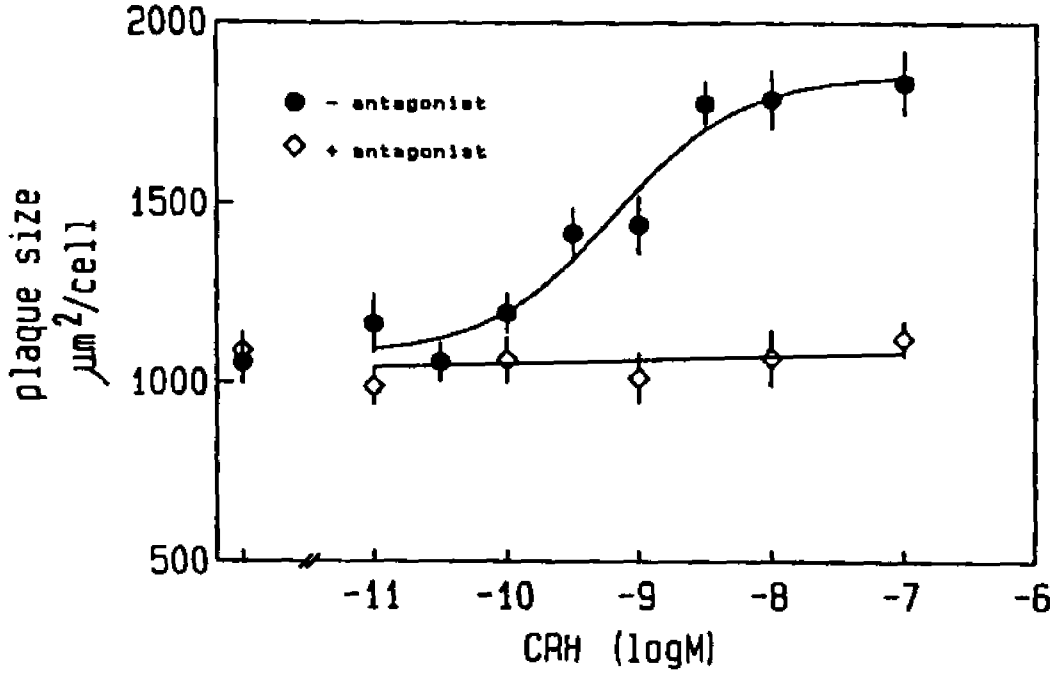

2000
1500
1000
500
plaque size
μm²/cell
● - antagonist
◇ + antagonist
-11
-10
-9
-8
-7
-6
CRH (logM)

Figure 5. Dose-response curve of CRH effects on the percentage of plaque-forming Leydig cells. Values in the top curve represent the average of two determinations made from at least 150 cells/chamber in duplicates. Values in the lower curve (+ antagonist) represent the percentage of plaque-forming cells in a chamber. The responses to CRH and the CRH antagonist in this experiment are representative of at least three separate RHPAs.

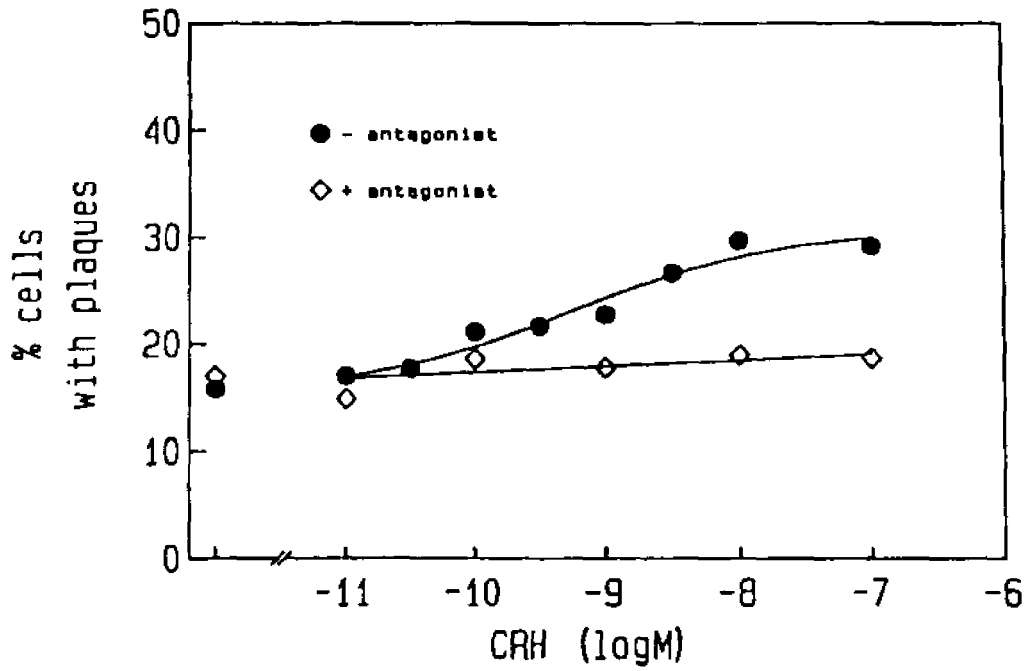

50
40
30
20
10
0
% cells
with plaques
● - antagonist
◇ + antagonist
-11
-10
-9
-8
-7
-6
CRH (logM)

TABLE 1

Effects of CRH and α-helical CRH-(9-41) on the percentage of total-forming cells

Treatment groups	Antagonist [α-helical CRH-(9-41)]	
	-	+
Basal (no CRH)	12.8 ± 1.6 (5)	12.0 ± 1.7 (3)
CRH (10^{-9} M)	19.9 ± 2.0 (4)[a]	12.2 ± 1.9 (3)
CRH (10^{-7} M)	20.1 ± 2.1 (4)[a]	12.9 ± 2.0 (3)

Numbers in parentheses indicate the number of determinations. Values are the mean ± SE. Two-way analysis of variance, followed by Student-Newman Keuls multiple comparison test, were performed. The level of significance chosen was $p < 0.05$.

[a] Values significantly different from basal levels without antagonist.

in the presence of the antagonist. Other results, in which immunostaining was not done, yielded results indistinguishable from those shown in Figure 4.

Figure 5 shows a separate analysis of the same experiment, in which the percentage of immunostained plaque-forming cells was plotted. There was a significant CRH concentration-dependent effect on the cells ($p < 0.05$) by this criterion also. Here again we note that a substantial fraction ($\sim 15\%$) of the Leydig cells formed plaques in the absence of CRH. In the presence of the secretagogue, only an additional 10-15% of the cells formed plaques.

Table 1 summarizes the results from several experiments.This analysis ignored the immunostaining, so that data from several experiments, with and without immunocytochemistry, could be analyzed together. The percentage of total plaque-forming cells in the presence or absence of CRH and the antagonist was calculated. Two-way analysis of variance showed a significant difference between the groups treated with CRH alone and all other groups ($p = 0.006$). From these results, we can further confirm that CRH causes a significant increase in the number of plaque-forming cells after 2 h of treatment, and that the antagonist inhibits CRH's effect.

5. DISCUSSION

POMC-derived peptides have been identified in testes of several mammalian species (Tsong et al., 1982), and a POMC mRNA capable of encoding the secretory prohormone has been found in human (Lacaze-masmonteil et al., 1987). In addition, biochemical and physiological responses to several POMC peptides have been shown to occur in this organ (Fabbri et al., 1985; Orth, 1986; Bardin et al., 1987; Knotts et al., 1988). From these findings has come the suggestion that POMC peptides synthesized in testes may have paracrine and/or autocrine functions in the male gonads. Implicit in this suggestion is the assumption that POMC peptides are actively secreted in a regulated fashion from the testicular cells in which they are made. Recently Fabbri *et al.* (1988) elegantly demonstrated selective hormonal stimulation of irβEP secretion from cultures of fetal Leydig cells while Valenca and Negro-Vilar (1986) demonstrated regulated secretion of irβEP from the adult whole testes in vivo and in vitro. Attempts to identify secreted POMC-derived peptides from testicular cells in culture by classical RIA appear to have been thwarted by proteases possibly secreted principally from Sertoli cells in the cell preparations (Fabbri et al., 1988) (Bardin, W., personal communication).

We focused our analysis specifically on βEP, because its presence and putative functions in the testis have been the most extensively documented of the POMC peptides (Fabbri et al., 1985; Orth, 1986; Bardin et al., 1987; Knotts et al., 1988). Moreover, we restricted our analysis to Leydig cells, because this cell type contains

βEP (Tsong et al., 1982; Bardin et al., 1987), while the germ cells, which also express the POMC gene, appear to cleave the protein to the shorter α and γ forms of the molecule (Cheng et al., 1985; Lebouille et al., 1986). Little, if anything, is known about the physiological roles of these forms of EP.

The RHPA, used in conjunction with immunocytochemical identification of Leydig cells, allowed us not only to study stimulated secretion of irβEP, but also to assess whether there is heterogeneity among Leydig cells in their capacities to secrete the hormone and respond to a secretagogue. We chose CRH as the secretagogue to test because this peptide is a potent stimulator of POMC peptide secretion from the pituitary (Antoni, 1986), and recent studies have suggested that CRH may also be synthesized locally within the testis (Thompson et al., 1987; Yoon et al., 1988).

Our results have demonstrated that a subset (>10%) of Leydig cells secretes a low but reproducibly detectable amount of irβEP in the absence of the test secretagogue. CRH increases the rate of irβEP release from these cells, but, as indicated by result of the time-course study, it does not seem to recruit new cells into this population of secreting cells. As shown in Figure 2, CRH increases the amount of irβEP released per cell up to 4 h (the longest time point studied), indicating that the cells remained viable and capable of responding to CRH for several hours. Whether the CRH-dependent increase in plaque size over several hours reflects an effect of the hormone on de novo POMC biosynthesis as well as secretion remains to be determined. As shown in Figures 4 and 5, the effect of CRH on irβEP occurs in a dose-dependent fashion, with a half-maximal response at about 0.7 nM CRH, which

is similar to the dissociation constant of 0.76 nM for CRH in the anterior pituitary reported by Wynn *et al.* (1983). Moreover, CRH stimulated secretion is completely inhibited by the CRH antagonist, suggesting that CRH is working through a specific receptor. Hence, the Leydig cell response to CRH is similar to that produced by primary cultures of pituitary corticotrophs with respect to both the concentration of CRH giving a half maximal response and the ability of the α-helical CRH-(9-41) antagonist to inhibit the stimulation (Rivier,J et al., 1984). Whether the two cell types make use of identical intracellular mechanisms for CRH stimulated POMC peptide secretion remains to be determined.

The mechanisms underlying the observed hererogeneity of Leydig cell irβEP production and CRH responsiveness are unknown. Heterogeneity in other biochemical markers of Leydig cells has been noted (Purvis et al., 1979). An obvious morphological heterogeneity in the testis is that of the waves of spermatogenesis occuring within individual seminiferous tubules (Pilsworth et al., 1981). Indeed, biochemical differences among Sertoli cells isolated from tubule segments containing sperm at different stages of differentiation have been noted (Ritzen et al., 1981). Perhaps, quantitative or qualitative differences among paracrine signals released into the interstitium from tubular cells of these various stages cause the heterogeneity among the Leydig cells.

Local CRH/POMC interaction in other tissues have also recently been documented. For example, CRH gene expression and CRH-stimulated POMC peptide secretion has been shown to occur in the human placenta (Petraglia et al., 1987b; Margioris et

al., 1988). If, similar to POMC gene, there is but a single CRH receptor gene per haploid genome in mammals, it may be hypothesized that the POMC gene and CRH receptor gene share common sequence elements that result in their coordinate expression in the same cells.

III. CORTICOTROPIN-RELEASING HORMONE STIMULATION OF β-ENDORPHIN SECRETION IN THE RAT TESTIS *IN VIVO*

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1. ABSTRACT

Corticotropin-releasing hormone (CRH) stimulates β-endorphin (βEP) secretion from adult rat Leydig cells *in vitro*. To evaluate the relevence of this action of CRH in a physiological context, we studied the effect of CRH on βEP secretion in the testis in whole animals.

In the current study, we measured the influence of locally infused CRH and synthetic analogs of it on βEP concentration in testicular interstitial fluid (TIF). in In all animals, the contralateral testis was infused with the saline vehicle alone, and thus served as a control for the other, peptide-infused testis. Following a 2 h infusion of a 60 μl volume, TIF was collected and assayed for βEP by RIA.

CRH (10 pmoles/min), CRH-(9-41) (10 or 25 pmoles/min), and the new CRH antagonist [DPhe^{12}, Nle^{21,38}, CαMeLeu^{37}]rCRH(12-41) (CRH-(12-41) (10 pmoles/min) did not alter TIF volumes compared to those of control testes.

In the pubertal rat, CRH stimulated irβEP levels in TIF approximately 3-fold ($p = 0.002$; $n = 9$, two tailed t-test, level of significance $p < 0.05$). The antagonist α-CRH-(12-41), infused along with CRH, resulted in TIF irβEP concentrations that were slightly but significantly lower than in paired saline controls ($p = 0.03$, $n = 7$). These results demonstrate that the intact testis of the pubertal rat is responsive to CRH. Moreover, they suggest that, at this developmental stage, endogeneously produced CRH may have a submaximal stimulatory effect on local βEP release.

CRH-(9-41) which, *in vitro* acted purely as an antagonist, in the intact pubertal

animal, had a small (1.6-fold) but significant stimulatory effect on TIF irβEP concentrations ($p = 0.04$, $n = 7$). This difference in action suggests the importance of chemical and/or structural microenvironment on the direction of response to the peptide hormone analog.

In adult animals, all the test peptides were without effect. These results, in conjunction with data from other studies demonstrating that testicular CRH levels are much higher in the adult than in the prepubertal animal, suggest that CRH production in adult testis is high enough to desensitize, and perhaps, down-regulate local CRH receptors.

The data presented here, in conjunction with result of several orther studies, demonstrate that βEP release from cells in the testis is a regulated process. Regulated secretion of the peptide implies that it is derived from a mRNA that contains a signal-peptide coding region. The readily detectable POMC-like RNAs in testis lack the 5'region found in the pituitary form of POMC mRNA, including the signal peptide coding sequence. Thus these short RNAs are not capable of encoding a precursor for peptides whose secretion is regulated. In the current study we document the presence of a low abundance mRNA that is indistinguishable from the pituitary form, and is therefore probably the source for POMC prohormone production in the testis.

2. INTRODUCTION

The testis is the site of synthesis of a wide variety of peptide hormones, including products of all three opioid peptide genes (Kilpatrick et al., 1986; Bardin et al., 1987; Garrett et al., 1989) and the so-called "stress-responsive" neuropeptide CRH (Thompson et al., 1987; Dunn et al., 1989). The stimulatory action of the hypothalamic CRH on POMC production and secretion in corticotrophs of the pituitary is well established in *vivo* and *in vitro* (Vale et al., 1981; Antoni, 1986; Lundblad et al., 1988). Therefore, we questioned whether there might be an analogous regulatory effect of CRH on POMC peptide within the testis.

In our initial studies in an *in vitro* system (i.e. removed from cell-cell interaction of the intact organ), CRH was found to stimulate irβEP secretion from primary adult Leydig cells (Eskeland et al., 1989) (chapter II). The effective concentration for half maximal response (EC_{50}) for this action of CRH was approximately 0.7×10^{-9} M, similar to the K_d of 0.76×10^{-9} M for CRH in the anterior pituitary reported by Wynn *et al.* (1983). The action of CRH was reversible by the CRH antagonist α-helical CRH-(9-41). These studies suggest the presence of a CRH/POMC stimulus/response system in the testis similar to that in the hypothalamus/pituitary axis.

A possible function for CRH in the intact testis has not been examined to date. Therefore, the current study evaluated the intratesticular action of CRH on irβEP secretion in the whole animal. In addition, we examined the effect of CRH on TIF,

which is a physiologically important compartment of the testis in which it acts as a vehicle through which factors are transported to regulate testicular function (Sharpe et al., 1983a).

It is well documented that POMC peptide expression and secretion in the testis are regulated (Chen et al., 1988; Valenca et al., 1986). A POMC mRNA containing a region that encodes for an amino terminal signal peptide is necessary for regulated secretory peptides (Gumbiner et al., 1982). The testis expresses a short POMC-like mRNA of approximately 900nt in length (Chen, 1987). This POMC mRNA which is orders of magnitude lower than in the pituitary, is about 200nt shorter than the full POMC transcript in the pituitary. It lacks exon 1 and 2 of the POMC gene and hence, the region that encodes for the amino terminal signal peptide present in the pituitary POMC mRNA (Jeannotte et al., 1987). Lacaze-Masmonteil *et al.* (1987) were able to detect the pituitary-like POMC mRNA in human testis. Only short POMC-like transcripts have been documented to date in the rat testis (Chen et al., 1984; Pintar et al., 1984; Gizang-Ginsberg et al., 1985; Bardin et al., 1987). Moreover, levels of POMC peptides in the testis are orders of magnitude lower than in the pituitary (Bardin et al., 1987). These testicular POMC peptides could be derived from low abundance full-length POMC transcripts. Therefore, we tested for the presence of a low abundance pituitary-like POMC mRNA in the rat testis.

3. MATERIALS AND METHODS

3.1 *Chemicals and supplies*

Iodogen for RIA was obtained from Pierce (Rockford, Il). CRH, α-helical CRH-(9-41) (CRH-(9-41)) and rat βEP were obtained from Peninsula Laboratories (Belmont, CA). The new CRH antagonist CRH-(12-41) ([DPhe$_{12}$, Nle$_{21,38}$, CαMeLeu^{37}]rCRH(12-41)) was the kind gift of Dr. Jean Rivier at the Salk Institute. Urethane, Tween20 and all other chemicals were purchased from Sigma (St. Louis, MO). Immulon 2 removable ELISA wells were obtained from Baxter (Edison, NJ). [^{32}P]CTP (800 Ci/mmole), [^{32}P]dATP and ^{125}NaI was purchased from New England Nuclear (Boston, MA). Reverse transcriptase was purchased from Seika Gaku (Florida); Taq polymerase was obtained from Cetus-Perkins Elmer (San Diego, CA). Nusieve was obtained from FMC BioProducts (Rockland, ME); and Seakem agarose was purchased from IBI (NewHaven, CT). Iodogen was purchased from Pierce (Rockford, IL). The βEP antiserum (EP-3) was the generous gift of Anthony Liotta (NIH), and has been extensively characterized (Liotta et al., 1981). This antiserum recognizes βEP, βEP-(1-27), α-N-acetyl derivative, and common precursor molecules.

3.2 *Animals, surgical procedure and treatment*

Sprague-Dawley male rats [40-45 days (pubertal) and 70-75 days of age (adult)] were purchased from Charles River Laboratories (Wilmington, MA). Rats were housed in a temperature and light controlled room (12 h on/12 h off) with free access

to food and water for up to 2 days before use. The rats were anesthesized with urethane (1.3 g/Kg, i.p.), and a small incision was made in the scrotum. Direct intratesticular infusion of test substances was performed by cannulation of the testis using a 25 gauge needle connected to a PE-50 tubing. A microsyringe (Hamilton) mounted on an infusion pump (Gage) was used to infuse test substance over a 2 h period at a rate of 0.5 μl/min. Assuming total TIF in the rat testis is 100 μl (not more that 10% testicular weight) and that all the peptide is retained in the testis (no clearance), the maximum concentration of each test peptide after a fifteen minute infusion is approximately 1.5 nM (10 pmoles/min) to 4 nM (25 pmoles/min) in the testis. In each animal, the test substance was delivered in a saline solution containing 5 mg/ml each of BSA and ascorbic acid. The contralateral testis was infused with saline concurrently to serve as a control.

TIF was collected as described by Sharpe et al. (1983b) and Valenca et al. (1986) with some modifications. After the animals were decapitated, the testes were removed and a small incision was made in the caudal end of the testicular capsule, and each testis was placed inside a syringe barrel on a nylon mesh bed. TIF was allowed to drain overnight at 4°C into a microcentrifuge tube containing 10 μl of 0.1 M acetic acid. Residual TIF was recovered by centrifugation at 400 x g for 10 min. TIF was then spun at 1000 x g at 4°C for 10 min and the supernatant was collected and its volume was measured.

3.3 *Extraction and radioimmunoassay*

Aliquots of TIF were diluted in the RIA buffer (0.1 M potassium phosphate buffer, pH 7.5 containing 0.1% gelatin and 0.2% Tween-20). The samples were heated at 100°C for 5 min to precipitate denatured proteins, spun at 4000g for 10 min and the supernatants were used in the assay.

Iodination and Plate RIA were described by Maidment et al. (1989). Briefly, 12 x 75mm tubes were coated with 50 μl of iodogen in methylene chloride. The solvent was evaporated under a stream of nitrogen, and the tubes were stored dry at 4°C until needed. Carrier-free iodine was added to an iodogen-precoated tube containing βEP dissolved in 0.1 M potassium phosphate, pH 7.0. After 10 min the reaction mixture was diluted to 1-1.5 ml with 0.01 N hydrochloric and 0.10 N acetic acid (HCl-HOAc) solution, and loaded onto a Sep-pak reverse-phase column pre-equilibrated in the HCl-HOAc solution. The peptide was eluted with a methanol gradient (0, 10, 30, 75, 90%) in the HCl-HOAc solution . Iodinated βEP was eluted with the 75% methanol solution. Five μg βEP/100 μCi ^{125}I was used.

In the plate RIA, Removawells were coated with 50 μl of 1 μg/ml protein A in 0.1 M sodium bicarbonate, pH 9.0, for 2 h at room temperature then washed 3 x 1 min with RIA buffer (0.2 M potassium phosphate, 0.1% gelatin, 0.2% Tween-20, pH 7.5). Buffer was added to the wells for another 30 min to remove any protein A that did not attach to the plate. Following 3 washes, 50 μl of βEP antiserum (1:2000) was added for 1 h. The wells were then washed 3 x 2 min and then 50 μl of βEP standards or samples were added overnight at 4°C. 50 μl of ^{125}I-βEP was then added for 2 h at room temperature. The wells were washed 3 x 2 min with ice-cold

buffer, detached and put in 12 x 75mm tubes, and counted for 5 min each in a gamma counter.

3.4 *RNA isolation, reverse transcription (RT) and polymerase chain reaction (PCR)*

Total RNAs from adult rat pituitary, testis, and Percoll-purified Leydig cells were isolated using the guanidinium method described by Ulrich *et al.* (1977). For the subsequent experiments the amount of total RNAs were 4 μg (pituitary), 10 μg testis, 3.5 μg Leydig cells, and 5 μg yeast RNA. RT was performed essentially as described by Wang *et al.* (1989). PCR amplification was performed following the Cetus-Perkins Elmers protocol with few modifications. In a 20 μl reaction volume, RT was performed on RNA samples (3 min denaturation at 95°C, 15 min annealing to 50 pmoles 3'end-primer at 55°C, and following the addition of 20 units of reverse transcriptase was added to each, samples were incubated for 30 min at 45°C). Ten μl of the samples were then directly amplified in a 50 μl volume. The conditions for PCR were: 0.5 min denaturation at 95°C, 0.5 min annealing with 100 pmole each of the 3' and 5'end primer at 55°C, 1 min elongation with Taq polymerase at 72°C, 45 cycles. The 5'and 3'end-primers were 26nt and 25nt in length, respectively. The 5'end primer comprised the sense strand nucleotides from nucleotides 4 to 29 in exon 1, while the 3'end primer region was the antisense sequence from nucleotides 8 to 32 from the translation initiation site ATG in exon 2 of the POMC rat gene as described by Jeannotte and coworkers (1987). The unlabelled reaction mixture was processed for Southern blotting and hybridization as described below. PCR amplification was

also done on the rest of the samples using radioactive dATP as a tracer so that the amplified products could be directly analyzed for size on a 6% acrylamide gel.

3.5 *Riboprobe synthesis*

The pXBex1 plasmid containing the POMC cDNA insert was kindly provided by J.L.Roberts. The POMC cDNA insert comprised all of exon 1 to a BamH1 site in Intron A of the POMC gene. The plasmid was linearized with Xmn1 restriction enzyme. The linearized plasmid was extracted, precipitated and dissolved in water. Radioactive cRNA copies were synthesized at 37°C following the Promega protocol, using SP6 polymerase. The riboprobe synthesis yielded transcripts of 97nt in length containing 37 nucleotides in the 3' end of exon 1 and a portion of the first intron.

3.6 *Southern transfer and filter hybridization*

Amplified DNA samples were loaded on a 3% NuSieve/1% Seakem gel. DNA was transferred to Gene Screen Plus filter (NEN, Boston) and hybridized with the riboprobe as described elsewhere (Maniatis et al., 1989), except that 400 mM Pipes, pH 6.7 was used instead of Tris buffer in the prehybridization and hybridization buffers. The filter was hybridized overnight at 42°C and washed twice with 2x SSC (1x: 0.15 M NaCl, 0.015 M Na citrate) at room temperature and twice at 37°C for 15 min. The filter was exposed overnight to X-ray film at -70°C.

3.7 *Statisitical analysis*

Comparison of irβEP levels in hormone-treated and untreated testes used paired t-test and Anova where appropriate. The level of signifcance chosen was $p < 0.05$.

4. RESULTS

4.1 The effect of CRH on irβEP levels in TIF *in vivo*

Dilution curves were obtained for irβEP in TIF of untreated pubertal and adult rats. The graph in Figure 6 shows dilution curves parallel to the standard curve, demonstrating that the antiserum recognizes a βEP-like peptide in the TIF of both pubertal and adult rats. The intra-assay coefficient of variation was less than 10%.

In the pubertal rat, CRH (10 pmoles/min for 2 h) caused an increase in the irβEP concentration in TIF by three-fold (Figure 7A) ($p = 0.002$). The competitive antagonist CRH-(9-41) (10 pmoles/min) also stimulated irβEP levels in TIF, as shown in Figure 7B ($p = 0.04$). When the pubertal animals were treated with CRH in the presence of the competitive antagonist CRH-(12-41) (10 pmoles/min), irβEP levels were, on average, below control (Figure 7C) ($p = 0.03$). Figure 7D is a summary of CRH effect in pubertal rats as percent irβEP stimulation.

In contrast to findings in pubertal animals, in the adult rat, neither CRH (10 pmoles/min), CRH-(9-41) (25 pmoles/min) nor CRH-(12-41) (10 pmoles/min) had any effect on irβEP levels in TIF as shown in Figures 8A, B,and C respectively ($p > 0.05$). Figure 8D shows the effect of CRH and its antagonists on the percent irβEP stimulation in adult rats.

TIF volumes in the pubertal and adult animals were unchanged when the different peptides were administered ($p > 0.05$). The average TIF volumes were 23.512 ± 2.831 (μl $\pm$ SE; $n = 23$) and 31.466 ± 4.813 ($n = 18$) in pubertal and adult testes,

Figure 6. Parallel analyses of serial dilutions of TIFs from pubertal and adult Sprague-Dawley rats measured by rat irβEP RIA. Std curve, synthetic rat βEP standard curve. B/B_0, bound to free ratio. The sample dilution curves have been displaced on the abcissa for display purposes.

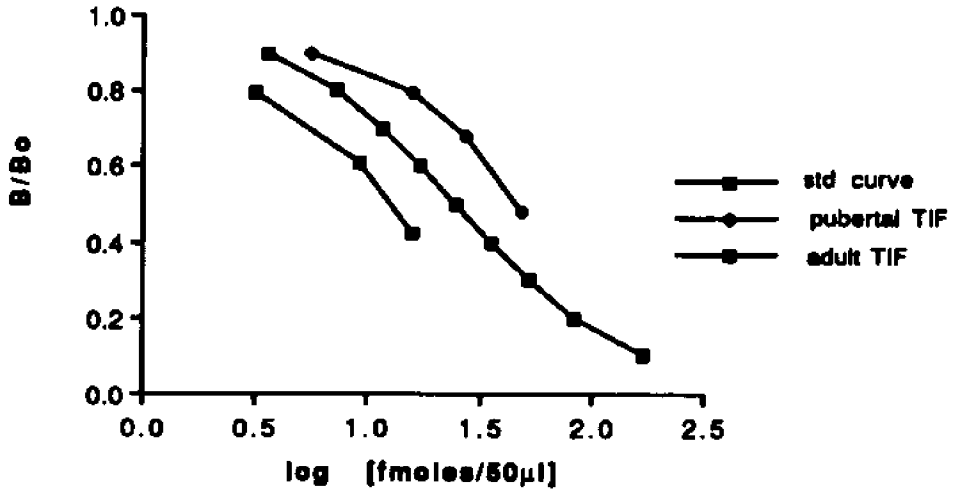
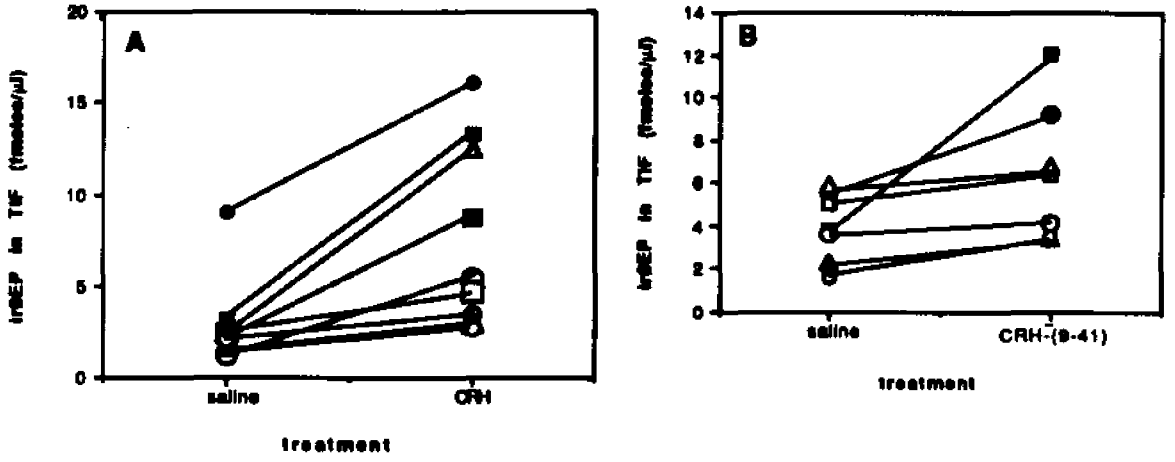
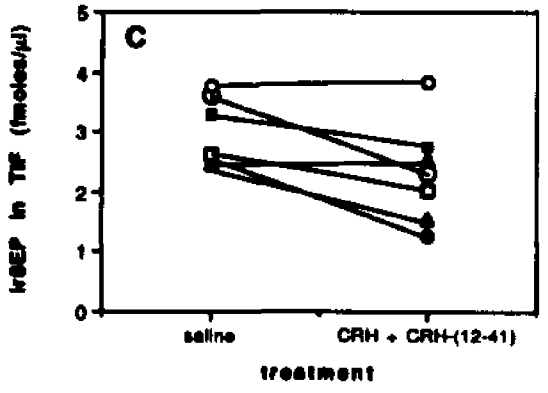

1.0
0.8
0.6
0.4
0.2
0.0
B/Bo
0.0
0.5
1.0
1.5
2.0
2.5
log [fmoles/50µl]
std curve
pubertal TIF
adult TIF

Figure 7. Effect of CRH and its antagonists on irβEP levels in TIF of pubertal rat. Each rat had one of its testes infused with saline as control. A, CRH *vs.* saline, n = 9. B, CRH-(9-41) *vs.* saline, n = 7. C, a mixture of CRH and CRH-(12-41) *vs.* saline, n = 7. D, Effect of CRH and its antagonists on the percent irβEP stimulation. Values in D are the mean ± SE (n = 7-9 animals/group). *, $p < 0.01$; **, $p < 0.05$.


A
B
hBEP in TIF (fmoles/µl)
saline
CRH
CRH-(9-41)
treatment


C
hBEP in TIF (fmoles/µl)
saline
CRH + CRH-(12-41)
treatment

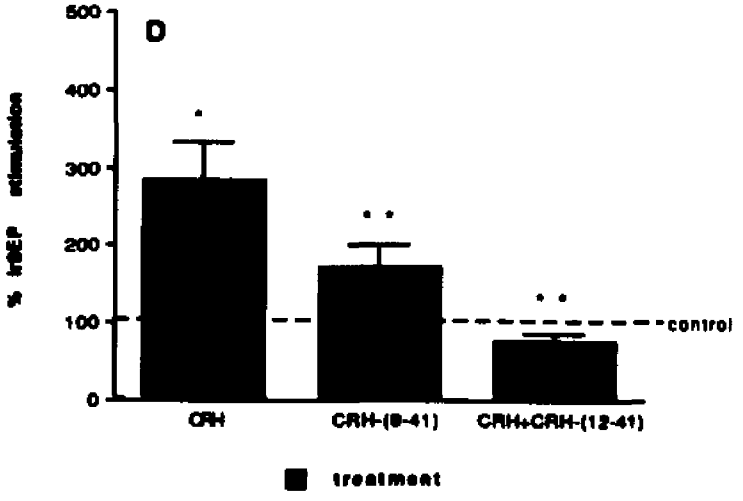
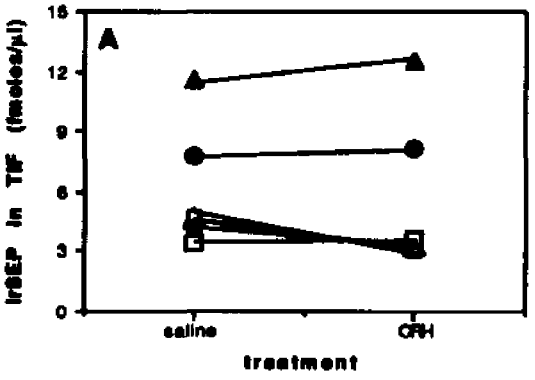
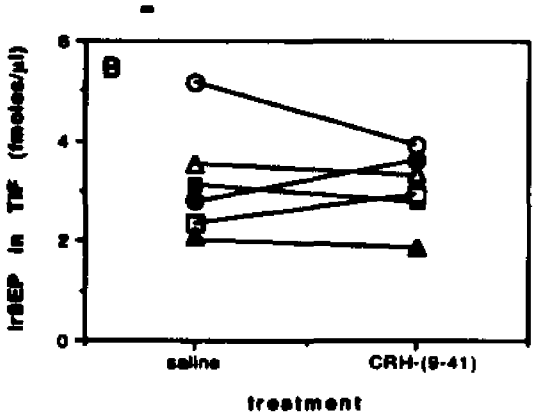
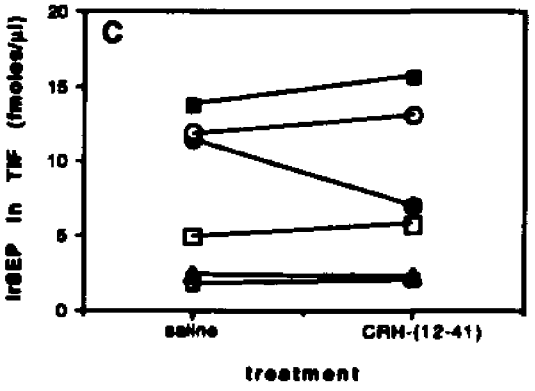
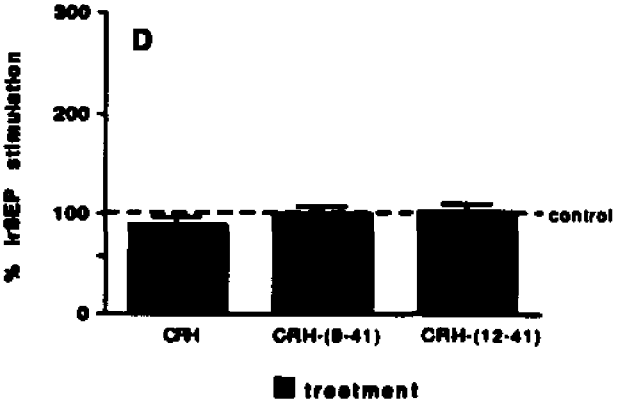

D
% hBEP stimulation
control
CRH
CRH-(9-41)
CRH+CRH-(12-41)
treatment

Figure 8. Effect of CRH and its antagonists on irβEP levels in TIF of adult rat. Each rat had one of its testes infused with saline as control.A, CRH *vs.* saline, n = 6 (n = number of animals). B, CRH-(9-41) *vs.* saline, n = 6. C, CRH-(12-41) vs. saline, n = 6. D, Effect of CRH and its antagonists on the percent irβEP stimulation. Values in D are the mean ± SE (n = 6 animals/group).


A
IrBEP in TIF (fmoles/µl)
15
12
9
6
3
0
saline
CRH
treatment


B
IrBEP in TIF (fmoles/µl)
6
4
2
0
saline
CRH-(9-41)
treatment


C
IrBEP in TIF (fmoles/µl)
20
15
10
5
0
saline
CRH-(12-41)
treatment


D
% IrBEP stimulation
300
200
100
0
control
CRH
CRH-(9-41)
CRH-(12-41)
treatment

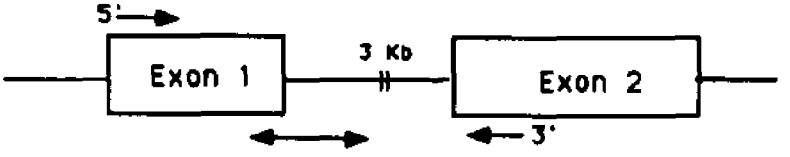
respectively.

4.2 Pituitary-like POMC mRNA in the rat testis

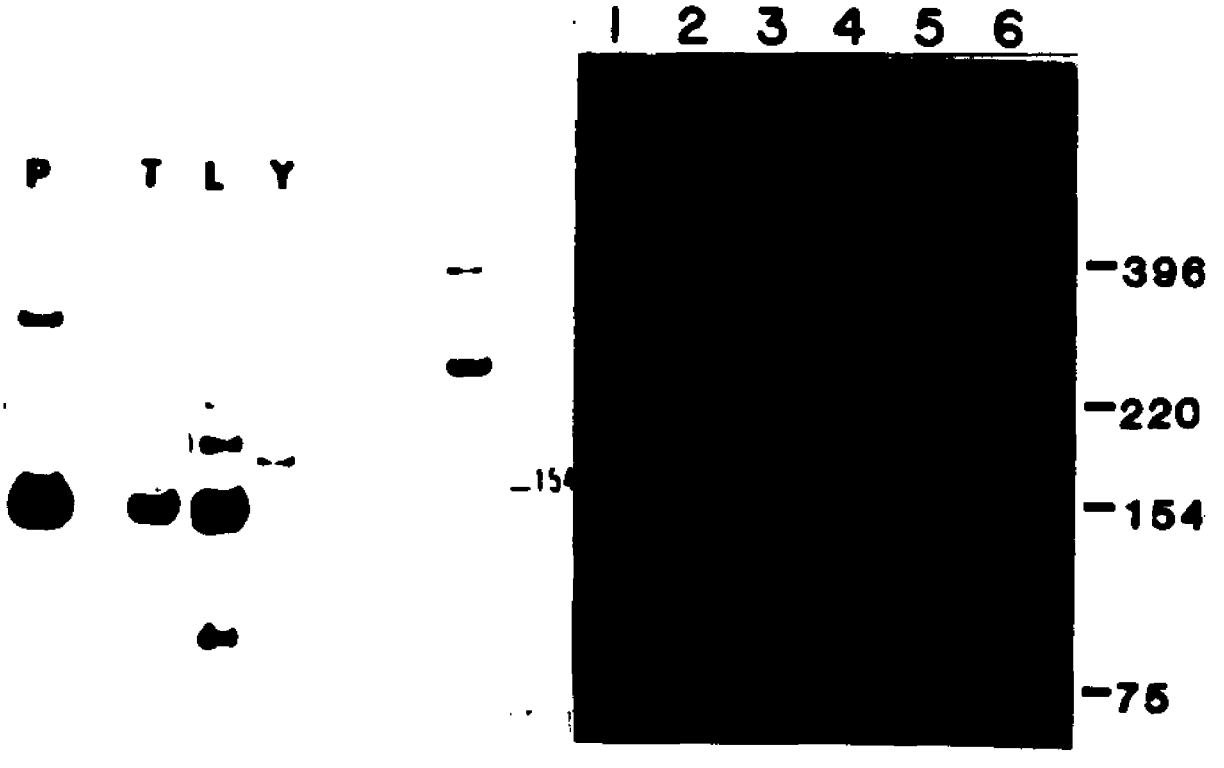
RNA samples isolated from adult rat testis and from an enriched adult Leydig cell preparation were analyzed for the presence of a pituitary-like POMC mRNA that could encode a prohormone for secreted peptides. To identify this RNA species, which if present, is in very low abundance, a sensitive detection method was used. First, cDNA synthesis was carried out on total RNA samples using an oligonucleotide primer complementary to a portion of the mRNA from exon 2, then the DNA product was itself copied using a sense-strand exon 1 primer. DNA products were amplified by repeated denaturation, priming, and synthesis. The expected size of the product generated from pituitary POMC mRNA is 147bp (Drouin et al., 1985). This is readily distinguishable from the ~3 Kb (kilobase) product generated from genomic DNA contamination in the RNA sample that could serve as a template in the amplification reaction.

To further verify that the 147bp band did indeed represent the 5'end of the POMC mRNA, unlabelled amplified DNA was subjected to Southern blot hybridization. The hybridization probe contained a 37bp sequence at the 3'end of exon 1, and therefore detected a product lying between, but not including the primers used for amplification. Radioactive amplified DNAs from adult rat pituitary, testis, Leydig cells, and yeast (negative control) were analyzed on acrylamide gel to determine the size of the amplified products. Pituitary, testis, and Leydig cells revealed bands that

Figure 9. Analysis of the RT/PCR products using [^{32}P] POMC riboprobe forhybridization, after Southern transfer. A, diagram of exons 1 and 2 of the POMC gene. The CRH riboprobe (↔) contains a 37nt region in Exon 1 and 60nt into Intron A. The 5'end primer (→) comprises the region from nucleotides 4 to 29 in exon 1, while the 3'end primer (←) comprises the region from nucleotides 8 to 32 from the translation initiation site ATG in exon 2 of the POMC rat gene. B, size analysis of the RT/PCR products. 22 μg of the amplified DNAs were loaded on a 6% acrylamide gel. The gel was exposed to x-ray film for 20 min with two intensifying screens. Lanes: P, pituitary; T, testis; L, Percoll-purified adult Leydig cells; Y, yeast RNA. Hinf1 cut PBR plasmid (marker) is in the far right lane. C, gel with RT/PCR products detected by hybridization with the riboprobe. The amplified products were extracted with phenol/sevag (chloroform:isoamyl alcohol, 24:1), followed by ethanol precipitation. The samples were dissolved in 50 μl TE (10 mM Tris, pH 8.0, 0.1 mM EDTA). 25 μl of the samples were loaded on the gel. Total rat RNAs were used in the experiments. Lanes: 1, pituitary, 4 μg; 2, Leydig cells, 3.5 μg (lost quite a bit in the loading); 3, testis; 10 μg; 4, yeast, 5 μg; 6, Hinf1 cut PBR plasmid as marker, 3 μg. The autoradiograph shown was exposed overnight to x-ray film.



A



B C

were 147 bp in length, which is the expected length for that region of the pituitary POMC mRNA (Figure 9B). Figure 9A shows the location of the primers and riboprobe in the POMC gene. Figure 9C shows the result of the Southern blot and hybridization with the riboprobe. Pituitary, testis and Leydig cells (the band is faint due to loss of sample during gel loading) show hybridization with the riboprobe. There is no band detected in the yeast lane.

5. DISCUSSION

We conclude that CRH can have a local physiological effect on irβEP secretion in the pubertal rat testis. That CRH is acting through specific receptors is suggested by the fact that irβEP release is stimulated following infusion of CRH, and by the response to the the antagonist CRH-(12-41) in blunting CRH's effect in the pubertal rats. In addition, the data suggest the presence of an endogenous CRH since the antagonist was able to decrease irβEP levels to lower than basal. Ulisse *et al.* (1989) have shown CRH binding sites in Leydig cells and CRH inhibited chorionogonadotropin hormone-stimulated testosterone levels in primary Leydig cell cultures. In addition, CRH stimulates irβEP secretion in adult primary Leydig cell cultures and immunoreactive CRH is localized in the interstitial and germ cells. These data suggest that CRH is acting on Leydig cells to stimulate secretion of irβEP in TIF. It is still possible, however, that the germ cells, through unknown mechanisms, may also respond to CRH stimulation of their βEPs secretion into the interstitium.

POMC peptides and CRH have been shown to be developmentally regulated in the testis. In the mouse and hamster, POMC peptides, which are under luteinizing hormone control, remain at low levels before puberty and increase to maximum after puberty (Bardin et al., 1987). In addition, irCRH levels in the testis of the rat is high before puberty, drastically drops at the onset of puberty and rises to maximum during adulthood (Yoon et al., 1988). Hypophysectomy drastically decreases testicular

irCRH levels (Yoon et al., 1988).

Our results demonstrated that CRH and its antagonist CRH-(12-41) were able to affect irβEP levels in TIF in the pubertal rat but not in the adult. This difference may be due to various factors during testicular development that can modulate the behavior of CRH at different stages of testicular growth. This factor(s) may affect CRH synthesis or its receptor.

Developmental changes in hormone sensitivity as a consequence of changes in amounts of specific receptors have been documented for a few specific peptides. For example, luteinizing hormone and arginine vasopressin have altered receptor number and function at different ages of the rat testes (Audhya et al., 1987; Tahri-Joutei et al., 1989b). It has been reported that seminiferous tubules at different stages of their cycle can influence Leydig cell morphology and function and gonadotropin binding (Risbridger et al., 1981; Sharpe et al., 1990).

Our results showing that CRH and its analogs had no effects on TIF βEP levels in the adult rat were unexpected, given that adult rat Leydig cells are quite responsive to CRH *in vitro* (Eskeland et al., 1989). The methodological explanation for the lack of such effects in the adult such as inactive CRH, was ruled out in three ways: 1) most of the individual experiments tested both pubertal and adult rats in parallel; in these studies the pubertal animals were responsive. 2) The bioactivity of the CRH batches was further confirmed by its ability to stimulate βEP secretion from AtT20 cells (CRH-responsive POMC-producing mouse pituitary tumor cells). 3) The CRH batches were further analyzed on HPLC for their intactness. Therefore, we speculate

that the factors that could play an important role in cell-to-cell communication in the intact testis are removed in culture systems with serum-free media. A similar phenomenon has been observed for vasopressin in which it was able to stimulate steroidogenesis production in adult Leydig cell cultures but not in the intact testis, when injected intratesticularly (Sharpe et al., 1987). Similarly, oxytocin was able to inhibit hCG-stimulated testosterone production by mixed testicular cells (Adashi et al., 1984), while *in vivo*, it had no effect (Sharpe et al., 1987).

It is of interest to point out that the CRH antagonist CRH-(9-41) acted as a weak agonist in the current *in vivo* studies while in primary adult rat Leydig cell cultures, CRH-(9-41) acted as an antagonist (Eskeland et al., 1989)(chapter II). Winslow *et al.* (148) reported agonist effects for CRH-(9-41) in behavioral experiments on monkeys. Due to different environments in the two systems, cells may be more sensitive to certain agents in intact organ versus dispersed cells.

TIF volumes have been reported to alter in response to LH and LHRH (Sharpe et al., 1983b; Valenca et al., 1986), suggesting that gonadotropins may play a role in osmoregulation and capillary wall permeability in the testis. Our studies suggest that neither CRH, nor its antagonists play a role as osmoregulators or regulate capillary wall permeability.

Our POMC mRNA results demonstrate that the rat testis, including the Leydig cells, have a low but detectable levels of pituitary-like POMC mRNAs. The short POMC-like mRNA detected in the rat testis by different investigators mentioned earlier is capable of being translated into a protein that recognizes βEP antisera

(Clark et al., 1990). This short transcript is missing the signal peptide coding region. Recently, Clark and coworkers (1990) demonstrated in transfection studies using GH_3 cells, the cells expressing the full-length mRNA secreted irACTH and irβEP. Cells expressing the short transcript secreted neither of these peptides. Their results suggest that the full-length POMC mRNA may be necessary for normal peptide processing and secretion. It is possible that the POMC protein cannot be further processed for storage and secretion; therefore, our results suggest that the irβEP detected in TIF may come from the long pituitary-like POMC mRNA in the testis.

In conclusion, these studies indicate that CRH may be important in maintaining normal testicular reproduction. Further studies must be conducted to understand the mechanisms and conditions in which CRH is involved locally in regulating steroidogenesis or spermatogenesis *in vivo*.

IV. THE EFFECT OF TEMPERATURE ON THE ACTION OF CRH ON IRβEP SECRETION IN TWO CELL LINES: TM3 AND AtT20

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Departments of Pharmacology (N.L.E.), Obstetrics, Gynecology, and Reproductive Science, and Anatomy (B.S.S.), Mount Sinai Medical School, New York, N.Y.

1. ABSTRACT

Testicular function is sensitive to chemical and thermal stresses. CRH and POMC, two peptide hormones that are involved in the body's neuroendocrine stress-response system are produced in the testis. We have recently documented that CRH stimulates secretion of βEP, a POMC peptide, from adult primary rat Leydig cells. To investigate the effects of small temperature changes on the CRH/POMC system using the reverse hemolytic plaque assay, we employed TM3 cells, a mouse prepubertal Leydig cell line that secretes irβEP and responds to CRH. In cultures treated with βEP antiserum (diluted 1:50) and complement (diluted 1:25), ~ 14% of the cells formed plaques. Increasing concentrations of CRH increased mean plaque area by 65% and percentage of plaque-forming cells by 60%. Very few plaques were formed around CRH-treated cells preincubated with βEP antiserum and βEP. The median effective concentration (EC_{50}) of the CRH dose-response curve at 34°C (testicular temperature in rodents) and 37°C (normal body physiological temperature) were 10^{-10} M and 10^{-9} M, respectively, for the total plaque area/100 cells. AtT20 cells, a pituitary tumor mouse cell line, were also treated with CRH at both temperatures. Using a radioimmunoassay to measure irβEP levels in the media, the EC_{50}s for the CRH dose-response curve at 34°C and 37°C were 2×10^{-10} M and 2×10^{-9} M, respectively, at 1 h. After 3.5 h the 34°C CRH dose-response curve shift was still observed. These results suggest that CRH receptors or post-receptor actions are sensitive to small temperature changes.

2. INTRODUCTION

The testes of many mammals reside in the scrotum, which serves as a thermoregulator that is apparently essential for normal male reproduction. The temperature of the scrotum is few degrees lower than the body's physiological temperature of 37°C. Temperature elevation from normal scrotal temperature to 37°C impairs germ cells (Chowdhury et al., 1970). Cryptorchidism (the translocation of testis to the abdomen) changes morphology and functions of germ, Sertoli and Leydig cells (Risbridger et al., 1981; Jegou et al., 1983; Lunstra et al., 1988). In the adult rat testis, hydrolytic enzyme activity (Blackshaw et al., 1978) and DNA synthesis (Fujisawa et al., 1988) decrease. In addition, DNA, RNA and protein syntheses in human testes are sensitive to temperature changes (Nakamura et al., 1988). Receptor number for gonadotropins, prolactin (Huhtaniemi et al., 1982), estrogen (Damber et al., 1983) and vasopressin (Tahri-Joutei et al., 1989a) are altered in response to thermal changes. Moreover, Leydig cells undergo hypertrophy and are hyperresponsive to luteinizing hormone-mediated testosterone secretion *in vitro* (Iturriza et al., 1979; Risbridger et al., 1981).

CRH, the potent hypothalamic hormone (Vale et al., 1981), is also made in the testis (Thompson et al., 1987; Yoon et al., 1988; Audhya et al., 1989). Recently, we have demonstrated that CRH stimulates the secretion of irβEP, a POMC peptide, from primary rat Leydig cells where the POMC hormone is synthesized (Eskeland et al., 1989)(chapter II). We proceeded to determine the effect of temperature changes

on this CRH/POMC system to better understand the role of thermal stress on normal testicular function. In addition, we also tested a non-testicular cell line that has the CRH/POMC system in order to assess the effect of temperature on CRH action on POMC in general. TM3, a prepubertal non-tumor forming POMC-producing mouse Leydig cell line (Mather, 1980; Bardin et al., 1987), as well as AtT20, an anterior pituitary tumor mouse cell line that secretes POMC peptides and responds to CRH (Affolter et al., 1985; Rosendale et al., 1987), were used in the secretion studies.

We used the RHPA in our studies on TM3 cells since it allows for the detection of low levels of irβEP from individual cells. We employed the plate RIA to measure irβEP in media from AtT20 cells since these cells secrete large amounts of POMC-derived peptides. Plate RIA was chosen over conventional RIA because this assay requires fewer steps and is very sensitive.

3. MATERIALS AND METHODS

3.1 *Chemicals and supplies*

CRH, α-helical CRH-(9-41), human and rat βEP were obtained from Peninsula Laboratories (Belmont, CA); guinea pig complement, pencillin-streptomycin, sterile saline, Dulbecco's Modified Eagle's Medium (DMEM) and DMEM/HAM F12 were obtained from Gibco (Grand Island, NY); Staphylococcal protein-A, poly-*l*-lysine, BSA, chromium chloride hexahydrate, paraformaldehyde, potassium phosphate dibasic, sodium bicarbonate, gelatin, and Tween20 were purchased from Sigma (St. Louis, MO); sheep blood preserved in modified Alsever's solution was obtained from Rockland (Gilbertsville, PA); Immulon 2 removable ELISA wells were obtained from Baxter(). The βEP antiserum was the generous gift of A. Liotta (NIH), and has been extensively characterized (Liotta et al., 1981).

3.2 *Cell culture conditions*

TM3 cells (ATCC,) were cultured in a 1:1 DMEM/HAM F12 media containing 5% horse serum and 2.5% fetal bovine serum and streptomycin-penicillin (10 units penicillin, 10 μg streptomycin). AtT20 cells (D16-16), kindly provided by Dr. James Roberts, were maintained in DMEM medium with 10% fetal bovine serum and antibiotics. Both cell lines were incubated in a 95% air / 5% CO_2 humidified at 37°C. Experiments conducted at 34°C used cells that were pre-incubated overnight at 34°C.

For the experiments, TM3 cells were harvested at subconfluency with a rubber policeman. RHPA was done on these cells as described below. AtT20 cells were plated in a 24 well plate at a concentration of 3 x 10^4 cells/0.5 ml/well, and cultured for 4 days before use. On the day of the experiment, AtT20 cells were washed 2 x with serum-free DMEM media containing 0.1%BSA and antibiotics . The cells were preincubated at the desired temperatures for 1 h; then CRH at different concentrations was added. The cells were incubated for the times indicated. Media were collected and frozen at -70°C until assayed. Cells were then washed twice with saline and 500 μl of 0.1% sodium dodecyl sulfate was added to the cells to lyse them. The lysates were frozen at -20°C until DNA determination was done using the method described by Hinegardner *et al.* (Hinegardner, 1971).

3.3 *RHPA*

The RHPA was performed as previously described (Smith et al., 1986). During the 3.5 h incubation, chambers received hourly media changes to ensure the continued presence of undegraded CRH. After 3.5 h the complement was added and all samples were incubated at 37°C for 1 h. The reaction was stopped by infusing through the chambers 2% gluteraldehyde in saline. The slides were then stored in 0.1M phosphate buffer at 4°C.

3.4 *Plate RIA*

Iodination and Plate RIA was described by Maidment *et al.* (1989). Briefly, 12 x

75mm tubes were coated with 50 μl iodogen in methylene chloride. The iodogen was allowed to dry through a stream of nitrogen, and the tubes were stored dry at 4oC until needed. 5μg βEP /100 μCi ^{125}I was used. The iodination procedure and plate RIA were described in chapter III.

3.5. *Quantitative analysis*

For the RHPA, the analysis was performed using Axiomat Image Analyzer System, and the plaque areas were calculated using the Drexel analysis program (Feingold et al., 1986). In each experiment, chambers without antagonist were assayed in duplicate, while each condition with antagonist was tested in a single chamber. All measurements and statistical analyses were done as previously described (Eskeland et al., 1989) (chapter II).

For the plate RIA, statistical analysis was carried out by analysis of variance and student paired t-test. The level of significance chosen was $p < 0.05$.

4. RESULTS

4.1 The effect of CRH on irβEP secretion from TM3 cells

Three rodent Leydig cell lines were tested for production of secreted irβEP. Only the mouse TM3 cell line, but not the rat R2C or LC540 cell lines, produced detectable levels of secreted peptide. Therefore, the TM3 were used for all subsequent studies.

Secretion of irβEP from TM3 cells was examined in the presence of CRH with or without its competitive antagonist α-helical CRH-(9-41)(10^{-6}M) for 3.5 h. Parallel experiments were done at 34°C (normal testicular temperature) and 37°C (the body's physiological temperature). As seen in Figures 10A through 10D, in which the experiments were done at 37°C, increasing concentrations of CRH significantly increased mean plaque size by approximately 70% and the percentage of plaque-forming cells by 10-15% ($p < 0.01$). In contrast, cells cultured in the presence of the antagonist showed no CRH-related increase in either mean plaque area or the percentage of plaque-forming cells above the basal levels (no treatment) ($p > 0.05$). Similarly, Figures 10C and D, in which the incubations were done at 34°C, show a CRH dose response curve in which CRH significantly stimulated mean plaque area ($\sim$ 70%) and the percentage of cells forming plaques by 10-15% ($p < 0.01$). It should be noted that at 34°C, lower concentrations of CRH than those at 37°C were able to maximally stimulate the mean plaque area and the number of plaque-forming cells. The effect of 1×10^{-9} M CRH at 34°C was higher than the effect at 37°C (p

= 0.002). In addition, at 34°C, when the antagonist was added with CRH it significantly inhibited CRH's effect but not to basal levels at the highest CRH concentration (10^{-7} M).

For negative controls, very few plaques were seen in chambers tested with antiserum preincubated overnight at 4°C with 2 μg synthetic human βEP/μl antiserum. No plaques were seen in chambers lacking βEP antisera or complement.

Figure 1E shows CRH dose-response curves at 34°C and 37°C on the total plaque area (mean plaque area x percent plaque-forming cells) in 100 cells. At 34°C, the EC_{50} is 1 x 10^{-10} M while at 37°C, the EC_{50} is 1 x 10^{-9} M.

4.2 The effect of CRH on irβEP secretion from AtT20 cells

We next tested a non-Leydig cell line in which irβEP secretion is stimulated by CRH to see if the temperature sensitivity is restricted to cells of the testis. We chose AtT20 pituitary cell line since it is well established that these cells have CRH receptors and that CRH stimulates POMC peptide secretion from these cells (Rosendale et al., 1987). Figure 2A shows CRH dose-response curves on irβEP release to the media at both 34°C and 37°C for 1 h. The dose-response curve at 34°C gave an EC_{50} of 2 x 10^{-10} M and at 37°C the EC_{50} was 2 x 10^{-9} M. A quantitatively similar result was observed following a 3.5 h stimulation. Figure 2B shows differences at both temperatures, with EC_{50}s of 0.8 x 10^{-9} M, and 5 x 10^{-9} M at 34°C and 37°C respectively. Similar to results with TM3 cells, the effect of CRH at 1 x 10^{-9} M was higher at 34°C than at 37°C ($p = 0.006$; statistical analysis done

Figure 10. Dose-response curve of CRH effects on mean plaque size and the percentage of plaque-forming cells in TM3 cells. The RHPA was performed for 3.5 h at 37°C (A,B) and 34°C (C,D). A,C, values in the top curves represent the mean ± SE of determinations made from 40-60 plaques in duplicate chambers; values in the lower curves (+ CRH-(9-41)) represent the mean ± SE of determinations made from 20-30 plaques in a chamber. B,D, values in the top curves represent the average of two determinations made from at least 150 cells/chamber in duplicates; values in the lower curves (+ CRH-(9-41)) represent the percentage of plaque-forming cells in a chamber. The responses to CRH and the CRH antagonist in this experiment are representative of at least two separate RHPAs. E, effect of CRH on the combination of plaque area and percentage of plaque-forming cells at 37°C and 34°C; the values represent mean plaque area x the percentage of plaque-forming cells per 100 cells.

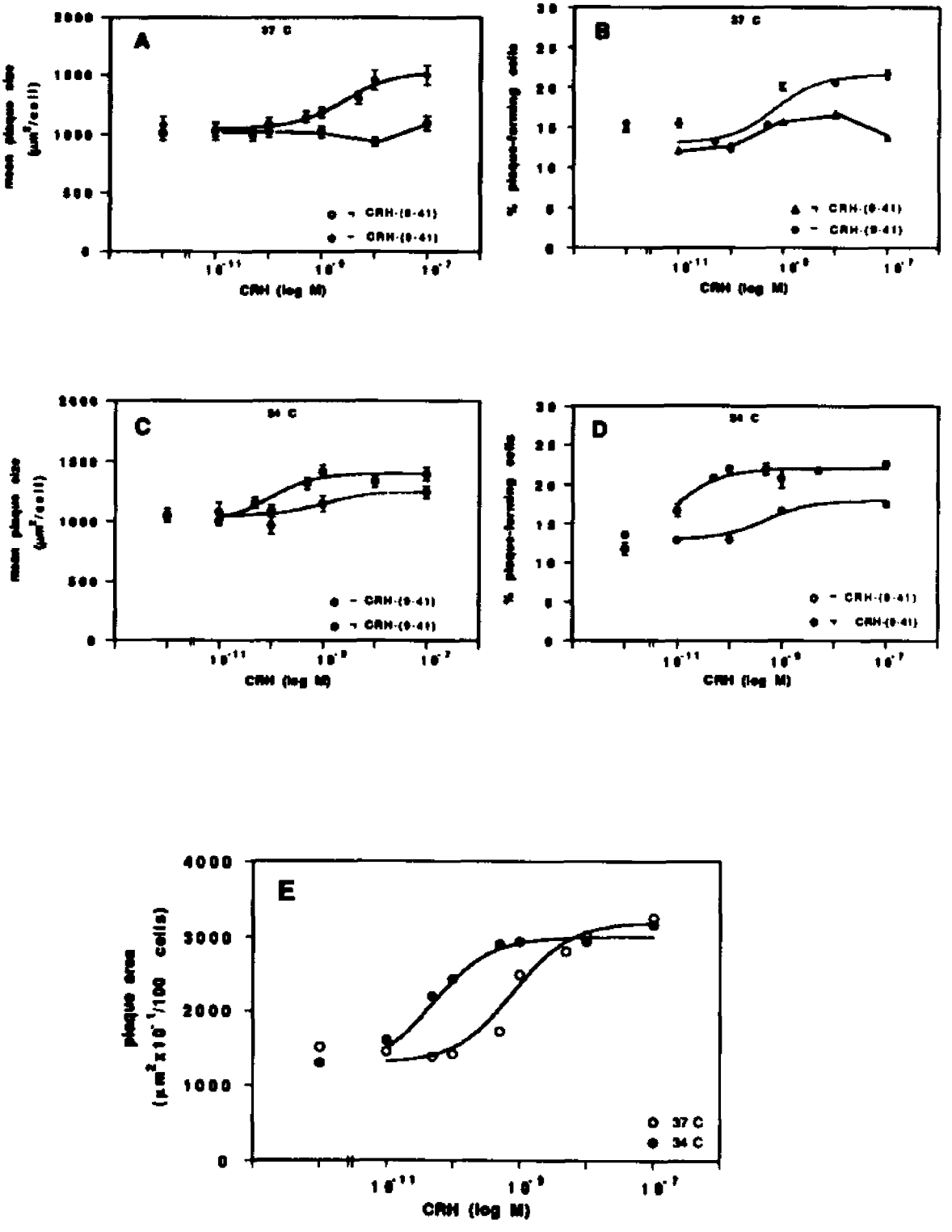
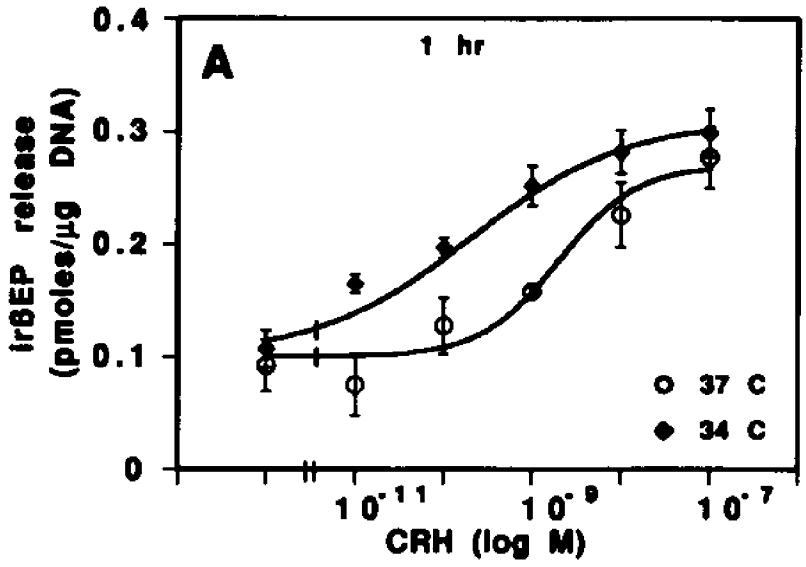
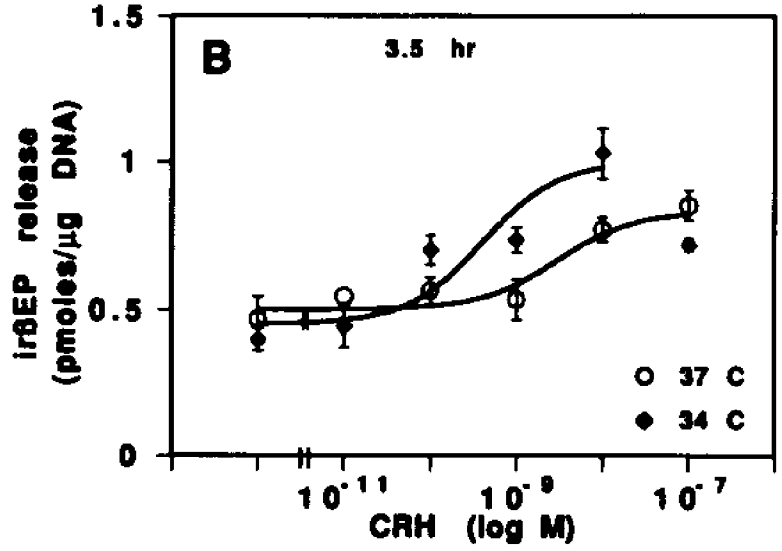

A
37 C
mean plaque size
CRH (log M)
B
37 C
% plaque-forming cells
CRH (log M)
C
34 C
mean plaque size
CRH (log M)
D
34 C
% plaque-forming cells
CRH (log M)
E
plaque area
(μm²x10⁻¹/100 cells)
4000
3000
2000
1000
0
37 C
34 C
CRH (log M)

Figure 11. Dose-response curve of CRH effects on irβEP release from AtT20 cells at 1 h (A) and 3.5 h (B) at 37°C and 34°C. Values represent the mean ± SEM of triplicate chambers. The 3.5 hr values are representative of at least three separate experiments.


A
1 hr
irBEP release
(pmoles/µg DNA)
0.4
0.3
0.2
0.1
0
37 C
34 C
10-11
10-9
10-7
CRH (log M)


B
3.5 hr
irBEP release
(pmoles/µg DNA)
1.5
1
0.5
0
37 C
34 C
10-11
10-9
10-7
CRH (log M)

on the combination of three separate experiments).

5. DISCUSSION

The data obtained provide evidence that TM3 cells actively secrete irβEP and that this action is specifically stimulated by CRH. Therefore, this cell line provided a convenient *in vitro* system for analyzing the temperature sensitivity of Leydig cell responsiveness to CRH.

The experiments were conducted for 3.5 h since these cells secrete low levels of irβEP and at shorter times the plaques are small and difficult to analyze. In RHPAs, the plaque size gets larger over time, an indication that the cells are viable and are continuously secreting the peptide of interest (Smith et al., 1986).

Both TM3 cells, a testicular cell line, and AtT20 cells, a pituitary cell line, showed greater sensitivity to CRH at 34°C than at 37°C. In the RHPAs with TM3, fresh CRH was infused into the chambers every hour to insure full CRH activity in our system because Ulisse and coworkers (1989) demonstrated that in adult Leydig cell cultures, about 50 % of the added CRH is degraded after a one hour incubation at 37°C.

The effect of temperature on CRH action was higher at 1 h than at 3.5 h (10-fold *vs.* 6-fold). These results suggest that the difference in sensitivity of cells to CRH is not merely due to more rapid degradation of CRH at the higher temperature.

Thermal regulation of the testis is critical for both testosterone production and spermatogenesis in the adult mammal. The cellular mechanisms responsible for testis sensitivity to small temperature changes are unknown. There is not much

documentation on tissues other than testes regarding cellular responses to effectors during hypothermia or hyperthermia. Vescovi and coworkers (1990) have shown in humans that during hyperthermia, POMC-derived peptide levels in the blood were raised, and the effect was selective since there was no corresponding increase in circulating levels of enkephalin. While in guinea pig, Nakane and coworkers (1985) reported CRH-dependent stimulation of ACTH levels in plasma in response to hypothermia. Our results suggest that CRH receptors or post-receptor mechanisms are sensitive to temperature changes. The observation made may only occur in dispersed cells and not in the intact organ, therefore, the physiological relevance of this phenomenon needs to be determined.

The EC_{50} for CRH dose-response curve at 34°C for the TM3 cells was similar to the k_d for CRH in Leydig cell membranes (1.3×10^{-10} M) reported by Ulisse *et al.* (1989), suggesting that 34°C may be the optimum temperature for the action of CRH in the testis. In the AtT20 data, the EC_{50}s at 34°C and 37°C after 3.5 h were higher than the EC_{50}s at 1 h. It is possible that some CRH degraded at both temperatures after 3.5 h.

The results of the full dose-response curves generated in our studies demonstrated the importance of such curves in determining the sensitivity of cells to agents under certain conditions. Using doses of drugs that would give maximum response is not sufficient to determine fully the effect of such a drug on target cells. Had we used only the maximum doses of CRH, we would have erroneously concluded that temperature changes have no effects on the action of CRH. Moreover, submaximal

concentrations are probably more physiologically relevant since endogenous modulators are themselves under regulation.

V. LOCALIZATION OF CRH mRNA IN THE TESTIS BY *IN SITU* HYBRIDIZATION

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1. ABSTRACT

The hypothalamic peptide CRH is the principal stimulator of the synthesis and secretion of POMC-derived peptides in the anterior pituitary. Both CRH and POMC gene expression have been documented in other tissues in the body such as the testis and placenta. Moreover, CRH has been shown to stimulate secretion of POMC peptides from testicular Leydig cells and from placental fragments cultured *in vitro*. Therefore, it appears that CRH serves a paracrine and/or autocrine function in regulating the POMC system in a number of organs.

In the rat testis, immunoreactive CRH has been localized, in one study, exclusively in interstitial cells, and in another study, in both the interstitium and in germ cells. However, the specific site(s) of CRH synthesis in this organ has not been determined. Therefore, we have investigated the site(s) of local synthesis of this peptide in the rat testis using the method of *in situ* hybridization to localize CRH mRNA. The probe used was a digoxigenin-labelled CRH riboprobe of approximately 700bp in length. Our studies showed an intense staining in the periphery of the tubules in which spermatogonia and primary spermatocytes reside. In addition, a number of tubules also had secondary spermatocytes and spermatids that stained. Positive staining was not obvious in Sertoli or interstitial cells. As a positive control, in the olfactory bulb, almost all cells stained as previously documented. Digoxigenin-labelled CRH sense strand RNA riboprobe showed no staining in either the testis or olfactory bulb. To further verify that the probe was specifically hybridizing to CRH mRNA, competition

studies were carried out: Sections that received excess unlabelled CRH riboprobe in addition to the labelled CRH riboprobe stained less strongly than those sections in which only digoxigenin-labelled CRH riboprobe was used. In addition, a section in which the labelled riboprobe was added with an excess of unlabelled estrogen receptor mRNA riboprobe (i.e. a heterologous RNA) showed staining intensity similar to the sections with only labelled CRH riboprobe. These results suggest that the germ cells are the predominant synthesizers of CRH mRNA.

2. INTRODUCTION

A number of peptides appear to have paracrine roles in the testis (Sharpe et al., 1987). For example, inhibin and LHRH-like peptide, which are primarily made in Sertoli cells, regulate testosterone production in Leydig cells (Sharpe et al., 1981, 1983b, 1987; Morris et al., 1988; Franchimont et al., 1989). POMC-derived peptides, which are synthesized in Leydig cells, primary spermatocytes and spermatogonia (Pintar et al., 1984; Cheng et al., 1985; Gizang-Ginsberg et al., 1985), regulate androgen-binding proteins and Sertoli cell proliferation (Boitaini et al., 1986; Orth, 1986). In addition, the hypothalamic peptide CRH is synthesized in the testis (Thompson et al., 1987), and it acts on Leydig cells to stimulate irβEP secretion (Eskeland et al., 1989)(chapter II) and to inhibit gonadotropin-stimulated testosterone release (Ulisse et al., 1989).

The site(s) of testicular CRH synthesis is unknown. IrCRH has been localized in interstitial and germ cells (Yoon et al., 1988). In cultures of enriched preparation of Leydig cells, irCRH is detected in the media as well as in the cell (Fabbri et al., 1990), suggesting that CRH is synthesized in these cells. In addition, translocation of the testis to the abdomen drastically decreases irCRH in the testis (Yoon et al., 1988). These reports suggest, but do not prove, that germ and Leydig cells of the testis are the source of CRH. In this paper, we attempted to determine possible origin(s) for CRH in the rat testis by using *in-situ* RNA/RNA hybridization technique to localize the site(s) of CRH mRNA.

3. MATERIALS AND METHODS

3.1 *Tissue preparation*

The procedure for tissue preparation and *in-situ* hybridization was previously described in Shivers *et al.* (1989) with few modifications: Testes and olfactory bulbs were removed from adult male Sprague-Dawley rat and frozen in crushed dry ice for 2 h. Tissues were mounted on microtome chucks using OCT compound (Optimum Cooling Temperature) and kept in a cryostat microtome at -15°C for two hours before cutting. Ten-micron sections were cut and adhered onto silanized (TESPA; Sigma) precleaned slides and transferred to clean slide boxes on dry ice until fixation time. The sections were then fixed for 5 min in 3 % paraformaldehyde in phosphate buffered saline in which diethylpyrocarbonate (DEPC, a ribonuclease inhibitor) (Sigma, St. Louis, MO) was added. The slides were then rinsed twice in PBS, and dehydrated in graded alcohol. The slides were stored with dessicant capsules at -70°C. Care was taken to insure that all solutions for fixation and in-situ hybridization were ribonuclease-free.

3.2 *Digoxigenin-labelled riboprobe synthesis*

The PGEM-3Z plasmid containing a CRH cDNA insert 759bp (bp, base pair) was the generous gift of Dr. Robert Thompson (University of Michigan). The CRH cDNA insert comprised the region of exon 2 of the rat CRH gene, from position

1227bp to postion 1986bp (Thompson et al., 1987). The plasmid was linearized with either HindIII (for antisense riboprobe) or EcoR1 (for sense riboprobe) restriction enzyme, extracted with phenol/chloroform, precipitated with ethanol and dissolved in TE (10 mM Tris, pH 8.0, 1 mM EDTA). For riboprobe synthesis in a 20 μl reaction, ~200 ng of the plasmid was used. Digoxigenin-UTP (DIG-UTP)-labelled probes were synthesized using the protocol described by Springer *et al.* (in press)(final concentration: 1x transcription buffer, 10mM DTT, 40 units RNAsin, 400 μM each of GTP, ATP, and CTP, 100 μM UTP, 400 μM DIG-UTP, 1 μl of a diluted [^{32}P]UTP [New England Nuclear] to monitor synthesis, and 19 units T7 RNA or SP6 Polymerase [Promega]). The synthesis was done for 2 h at 37°C followed by a 10 min, 37°C DNA digestion with 1 unit of DNAse and 40 units of RNAsin. Then, the mixture was adjusted to final concentration of 50 mM EDTA and 1x STE (1 M NaCl, 100mM Tris-HCl, pH 8.0, 10mM EDTA) , 13.2 mM DTT and 25 μg tRNA as carrier. The mixtures were ethanol precipitated, pellets were washed with 95 % ethanol and dissolved in 50 μl DEPC-treated water. Aliquots of the riboprobes before and after precipitation were loaded on a 4 % acrylamide gel to monitor the intactness of the riboprobes. Serial dilutions of the riboprobes were spotted on a nitrocellulose paper and processed for alkaline phosphatase detection (described below) to confirm that the DIG-UTP was incorporated in the riboprobes.

3.3 *In situ hybridization*

Sections were removed from the -70°C freezer and allowed to thaw in a dessicator

for 30 min. Clear, viscous nail-polish (Wet and Wild, Revlon) was used to form barriers between sections receiving different treatments. The sections were incubated in 2x SSC (1x : 150 mM NaCl, 15 mM Sodium Citrate, pH 7.0) with 5 μg/ml proteinase K for 10 min then rinsed twice in 2x SSC for 10 min. The slides were placed in a Nalgene utility box containing Whatmann 3MM filter paper saturated with 4x SSC, 50% formamide. Prehybridizaiton and hybridization buffers were described by Shivers and coworkers (Shivers et al., 1989) except that 20 mM Pipes, pH 6.7 buffer was used instead of Tris. Preheated prehybridization buffer was added to the sections, and the boxes were kept in a humidified chamber for 2 h at 50°C. The prehybridization buffer was removed from the sections, and preheated hybridization (same as the prehybridization buffer except for 0.005% yeast total RNA and 0.01% herring sperm DNA) solution containing either 300 ng/ml antisense or sense CRH riboprobe or no riboprobe. Some sections had, in addition to the antisense CRH riboprobe, excess unlabelled antisense CRH or estrogen receptor riboprobe (3 μg/ml) in the hybridization solution. Hybridization was carried out overnight at 50°C. The slides were then washed in 2x SSC for 45 min at room temperature. Thirty μg/ml RNase A in 0.5M NaCl, 10mM Tris pH 8.0 was added to the sections for 30 min. The slides were washed for 1 h in 2 liters 2x SSC at 50°C, 3 h in 4 liters of 0.1x SSC at 50°C followed by an overnight wash in the same solution at room temperature with gentle stirring.

3.4 *Detection*

The detection method was described in the Boehringer Mannheim protocol using the Dig Nucleic Acid Detection Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). After the overnight wash, the slides were incubated in 2x SSC, 0.05% Triton X-100 (Fisher Scientific, Orangeburg, N.Y.) containing 2% normal goat serum (Vector Laboratories, Burlingame, CA) for 2 h. The slides were then washed twice for 5 min in Buffer 1 (100mM Tris-HCL pH 7.5, 150 mM NaCl). The slides were placed in a box containing a Whatmann 3MM filter saturated with Buffer 1. Anti-digoxigenin-AP conjugate (diluted 1:400) in Buffer 1 containing 1% normal goat serum and 0.3% Triton X-100 was added to the sections and the box was placed overnight at 4°C. The slides were then washed once in Buffer 1 for 10 min followed by a 10 min wash in Buffer 2 (100mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). The sections were incubated with Chromagen (freshly prepared in a dark bottle by adding 0.338 mg/ml Nitroblue Tetrazolium salt (NBT), 0.175 mg/ml 5-Bromo-4-Chloro-3-Indolyl Phosphate, Tolidinium salt (BCIP), 2.4 mg levamisole in Buffer 2) in a light tight box with paper saturated with Buffer 2 for 2 h, until color was developed. The reaction was stopped by rinsing the slides in Buffer 3 (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). Sections were finally rinsed in water, nail polish was removed, and slides were coverslipped using Gel/Mount Tm (aqueous based mount, BIOMEDA Corp.). The slides were stored in a box at -20°C to minimize diffusion of the reaction product. At all times, care was taken not to let the sections dry out.

4. RESULTS

4.1 CRH mRNA in the testis

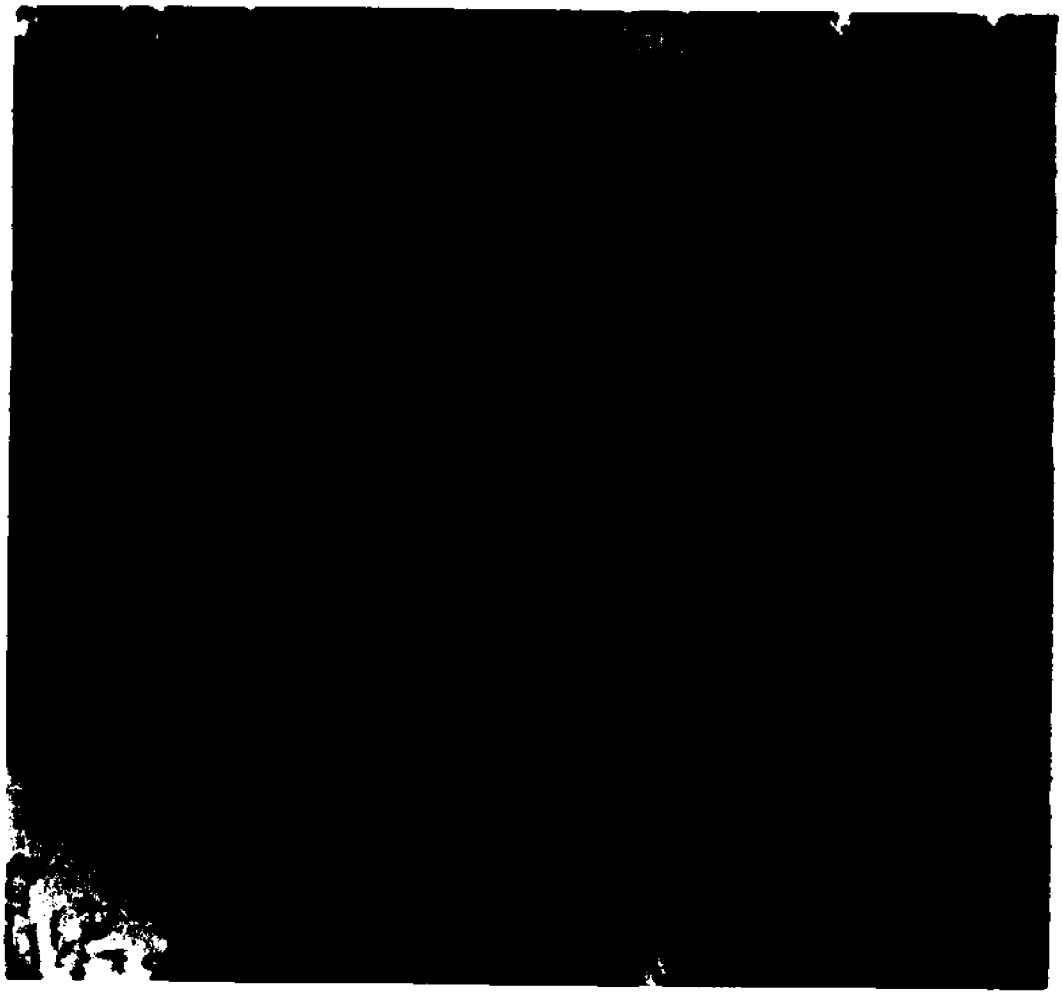
The digoxigenin-labelled CRH riboprobe showed intense staining of germ cells in the tubules as seen in Figure 12A. Figure 12B shows a negative control using digoxigenin-labelled sense strand CRH riboprobe. Only background staining is observed with that riboprobe. Figure 13A shows a higher magnification of the interstitial and germ cells. As shown in those figure, primary spermatocytes, spermatogonia and spermatids stained strongly with the antisense riboprobe; no staining was observed in the interstitial cells. The stained cells scattered in the interstitium appear to have the morphology of germ cells; these cells are probably from broken tubules.

4.2 Competition experiments and positive control

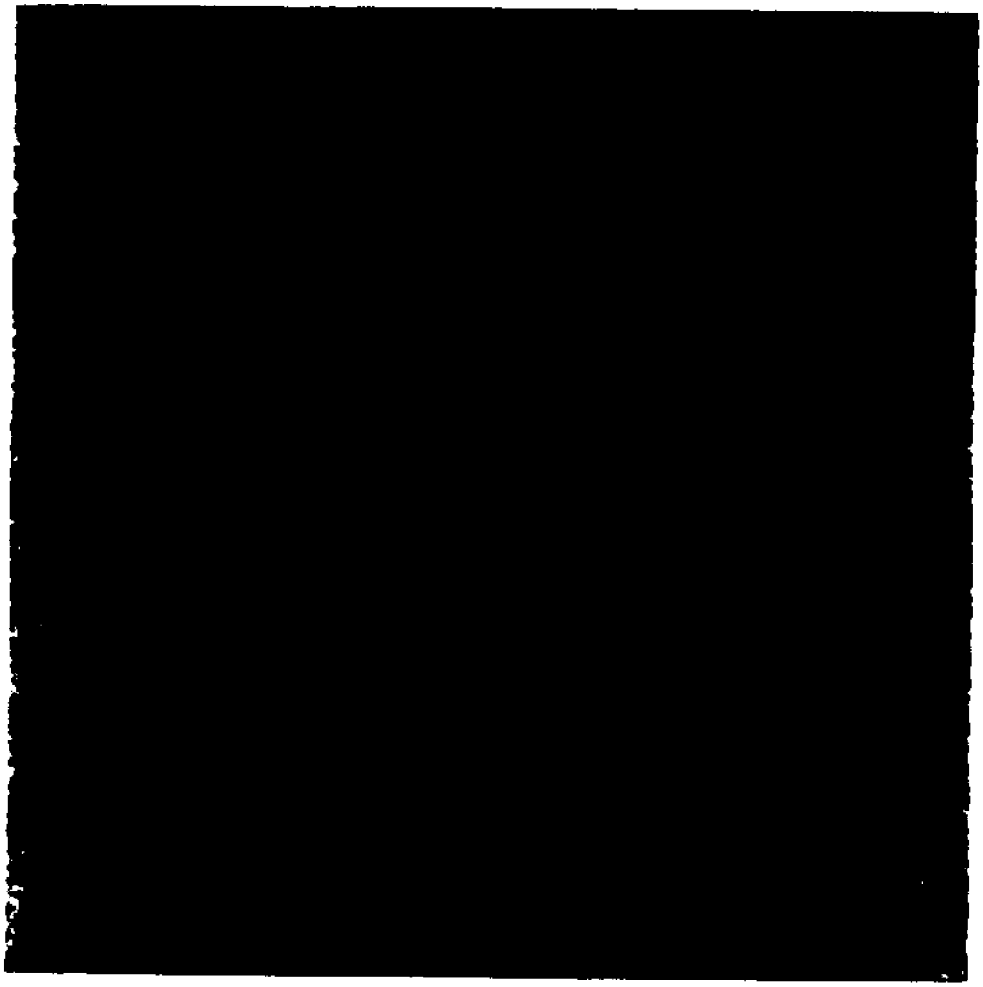
To further confirm that the riboprobe used was specific in its hybridizaiton to CRH mRNA, testicular sections received a 10-fold molar excess of unlabelled antisense CRH riboprobe or excess (10x) unlabelled antisense estrogen receptor riboprobe in addition to the digoxigenin-labelled antisense CRH riboprobes. Figure 13B, in which a section received the excess unlabelled CRH riboprobe, stained less than the section in Figure 13C, in which the tissue received excess unlabelled estrogen receptor riboprobe.

As a positive control for CRH mRNA detection, olfactory bulb sections were tested

Figure 12. *In situ* hybridization staining of CRH mRNA in an adult rat testis using digoxigenin-labelled riboprobe. A, antisense CRH riboprobe. B, sense CRH riboprobe. Nomarski optics; magnification, x160



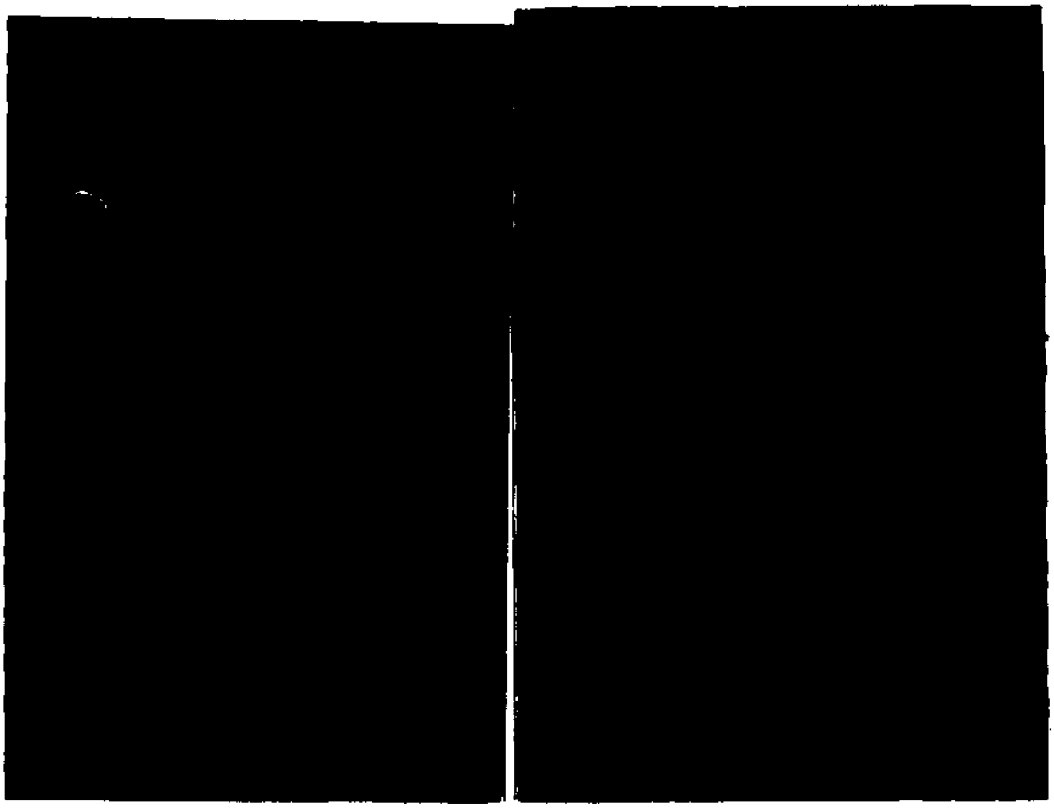
A



B

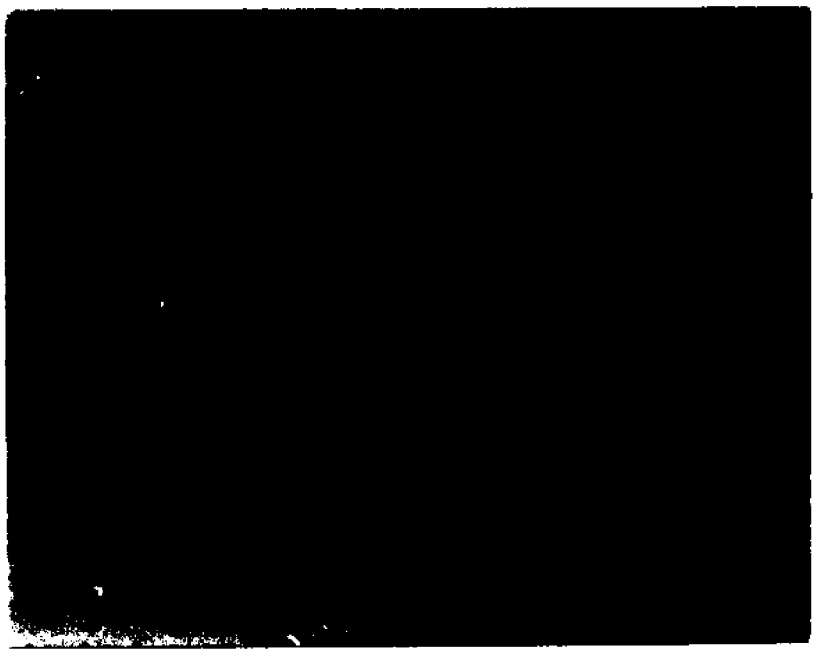
Figure 13. *In situ* hybridization staining of CRH mRNA in testis and olfactory bulb. A, interstitial cells of the testis and seminiferous tubules. ST, spermatid; SC, spermatocyte. Nomarski optics; magnification, x320. B,C, competition studies with digoxigenin-labelled antisense CRH riboprobe in a testis. B, excess unlabelled CRH antisense riboprobe and digoxigenin-labelled riboprobe in the testis. C, excess unlabelled estrogen receptor antisense riboprobe. D,E, olfactory bulb. D, antisense digoxigenin-labelled riboprobe. E, sense digoxigenin-labelled riboprobe. Bright field optics; magnification, x32.

A



B C

D



E

in the same experiment. Figures 13D and E show sections that were treated with either the antisense labelled CRH riboprobe or with the sense labelled riboprobe, respectively. Figure 13D is positive for CRH mRNA labelling, while figure 13E is negative.

5. DISCUSSION

CRH-like mRNA has been found in the RNA extracted from rat testis (Thompson et al., 1987) and immunoreactive-like CRH peptide has been demonstrated in testis, suggesting that the testis is capable of synthesizing this neuropeptide. Audhya and coworkers (Audhya et al., 1989) detected irCRH only in interstitial cells, while Yoon and coworkers detected irCRH in the germ cells as well (Yoon et al., 1988). These conflicting documentations may be due to differences in antibody recognition of a CRH-like peptide. Fabbri and coworkers (Fabbri et al., 1990) reported irCRH in the media from Percoll-purified adult Leydig cells in culture, again suggesting that these cells synthesize the peptide. Our non-radioactive *in-situ* hybridization results demonstrated that spermatogonia, spermatocytes, and spermatids are the major sites of CRH mRNA. The *in situ* hybridization may not have been sensitive enough to detect low abundant CRH mRNA in interstitial cells.

In our previous studies, in which immunocytochemistry was used to restrict our analysis to Leydig cells, the CRH antagonist α-helical-CRH-(9-41) did not lower basal irβEP levels, suggesting that the POMC-producing Leydig cells do not secrete CRH. In addition, Fabbri *et al.* (1990) reported irCRH levels in enriched Leydig cell preparations that were several hundred-fold lower than the irCRH levels in total testis extracts reported by Yoon *et al.* (1988). Assuming that there are approximately 10 million Leydig cells/ rat testis, the findings suggest that the major source of a CRH-like peptide in the adult rat testis is not Leydig cells but non-Leydig interstitial

cells and/or germ cells.

The riboprobe used in the *in situ* hybridization studies appears to be specific in its detection of CRH mRNA since excess unlabelled antisense CRH RNA was able to compete with digoxgenin-labelled riboprobe but excess unlabelled antisense estrogen receptor RNA (a heterologous RNA) was not able to compete. In addition, the digoxigenin-labelled sense CRH riboprobe gave negative results in both the testis and olfactory bulb tissues. The staining pattern in the olfactory bulb, using the digoxigenin-labelled riboprobe, was similar to the patterns seen in *in situ* hybridizations with radioactive CRH probes and immunocytochemical detection of CRH distribution in the rat olfactory bulb (Imaki et al., 1989).

Functional CRH receptors have been shown in enriched adult Leydig cell preparations. CRH stimulates irβEP secretion (Eskeland et al., 1989) and inhibits luteinizing hormone-dependent stimulation of testosterone secretion in Percoll-purified Leydig cells (Ulisse et al., 1989). In addition, CRH stimulates irβEP concentrations in testicular interstitial fluid in the whole animal at the pubertal age (NLE, CM, CC, BSS; chapter III). These reports suggest a local regulatory function for CRH in rat testis.

Cell-to-cell interactions in the testis are essential for the process of spermatogenesis. Different peptides such as oxytocin, inhibin, and LHRH-like peptide made in the Sertoli cells regulate the function of Leydig cells (Sharpe, 1984; Sharpe et al., 1986, 1987). In addition, depending on the stage of seminiferous tubules, Leydig cells have different morphology (Bergh, 1982); moreover, experimental cryptorchidism, which

depletes germ cells, alters Leydig cell function (Risbridger et al., 1981). The mechanisms by which cells communicate in the testis, especially communication between germ and interstitial cells are unknown.

VI. GENERAL DISCUSSION

A. CRH effect on irβEP secretion from Leydig cells of adult rat testis *in vitro*

CRH was able to stimulate irβEP secretion from Leydig cells in culture. The action of CRH was through specific receptors since the CRH antagonist α-helical CRH-(9-41) was able to inhibit CRH-dependent stimulation of irβEP release. The RHPA method used to study irβEP secretion, followed by immunocytochemical identification of Leydig cells, provided a powerful tool to discriminate between secreting and non-secreting Leydig cells, at least in an *in vitro* system. In Percoll-purified Leydig cell cultures, only a subset of the monoclonal stained-cell population secreted irβEP and responded to a CRH stimulus.

The reason for this heterogeneity is not clear but may reflect position-dependent differences among these cells at the time they were removed from the testis. The presence of a Leydig cell cycle in the mammalian testis has been suggested by the work of Bergh (Bergh, 1982). Bergh documented that some Leydig cells with a decreased cell density were preferentially associated with seminiferous tubules mainly in the stages of spermatid elongation and maturation (Leblond et al., 1952), while Payne and coworkers (Payne et al., 1980) found two Leydig cell populations that differed in their responses to LH. In addition, Gizang-Ginszberg and Wolgemuth (1985) found expression of a POMC-like mRNA in Leydig cells preferentially associated with the tubule stages described above. Sharpe *et al.* (1983b; 1984)

suggested that factor(s) from the tubules are responsible for differences in Leydig cell morphology and function. Therefore, given this functional heterogeneity among Leydig cells, it may be that only a subset have CRH receptors. Ulisse *et al.* (1989) found that in an enriched Leydig cell preparation, there were very low levels of CRH receptors per cell (300/cell). Perhaps the true receptor distribution in the tissue is very heterogeneous with only a subset of the cells having a much higher receptor concentration.

Staining for irβEP-like material was previously observed in the majority of interstitial Leydig cells (Bardin et al., 1987). Since we found that only a subset of Leydig cells secrete irβEP, it may be that many of these cells have the POMC peptides but do not secrete them, assuming that the RHPA is sensitive enough to detect minute secretions from the cells. Possibly the irβEP in most Leydig cells is the product of the short POMC mRNA that translates a non-secretable protein.

Fabbri *et al.* (1990) demonstrated reversal of endogenous CRH inhibition of hCG-stimulated testosterone secretion by employing either the antagonist CRH-(9-41) or an antibody specific to CRH. This endogenous CRH inhibition was only seen in cultured Leydig cells and not in cells in suspension. They postulated that cell-to-cell interaction would favor the availability of secreted CRH to specific receptors. This postulate is in agreement with our finding in Leydig cell cultures, in which the antagonist CRH-(9-41) could not inhibit basal βEP secretion in the RHPAs where there is no Leydig cell to Leydig cell contact.

B. CRH effect on irβEP secretion in the rat testis *in vivo*

The current studies on the whole animal demonstrated a physiological relevance of CRH on βEP regulation. CRH was able to stimulate irβEP secretion in TIF in the pubertal rat but not in the adult, suggesting a developmental change in sensitivity to CRH. This finding in the study of the adult was in contrast to our finding in studies on cultured cells from adult rats, in which CRH was able to stimulate irβEP secretion. Perhaps this apparent contradiction reflects the fact that factors that may play a significant role in regulating the action of CRH in the intact adult testis are removed in dispersed cells. This observed difference between cells in culture and in the intact testis has precedent since Sharpe and coworkers (Sharpe et al., 1980) demonstrated differences between dispersed Leydig cells and cells in intact testes with respect to their sensitivity to gonadotropin-stimulation *in vitro* due to altered LH receptor numbers. In addition, arginine vasopressin was able to stimulate testosterone secretion in cell cultures from adult testis, but had no local effect *in vivo* (Sharpe et al., 1987). Moreover, *in vivo*, the competitive antagonist CRH-(9-41) acted as a weak agonist, in contrast to our *in vitro* system. These results suggest that experimental manipulation as well as environmental conditions can affect Leydig cell sensitivity and response to certain agents.

The observed action of CRH and its antagonist *in vitro* versus *in vivo* system demonstrates the necessity of whole animal experiments. Culture systems may reveal the existence of specific functional receptors in cells but do not provide information about the direction or magnitude of hormone action in the intact organ.

We observed that, in the pubertal animals, the antagonist CRH-(12-41) when added with CRH, was able to decrease irβEP levels in TIF from controls, indicating the presence of an endogenous CRH-like peptide that is able to increase irβEP levels in TIF of the pubertal rat testis. It is not known what factor(s) contribute to altered responsiveness of CRH during development.

TIF is the only vehicle for hormone transport into the testis and between testicular compartments (Sharpe et al., 1983a). Experimentally, TIF provides a simple means of sampling the intratesticular environment and measuring factors that are believed to be of importance as intratesticular communicators or regulators (Sharpe et al., 1983b). TIF is also an index of changes in capillary wall permeability which are important in the regulation of the energy and uptake of important hormones in the testis. Sharpe and coworkers reported changes in TIF volume after subcutaneous LH or hCG administration (Sharpe et al., 1983b), while Valenca and coworkers reported TIF volume changes after intratesticular admininstration of LHRH and LH (Valenca et al., 1986). In our experiments, CRH did not affect TIF volume, indicating that this peptide does not play a role on capillary wall permeability in testis.

C. Pituitary-like POMC mRNA in the rat testis

The demonstrated presence of the long pituitary-like POMC mRNA in rat testis as well as in rat Leydig cells suggest that this transcript may be the message that encodes the POMC prohormone and hence the irβEP that is secreted into TIF in the intact testis and into the media in cell culture systems. It is still possible that tissues

such as the testis that contain the short POMC transcript have different processing enzymes as well as packaging requirements so that POMC-derived peptides can still be able to be stored in vesicles and be secreted in a regulated manner.

D. Temperature effect on the action of CRH

Maintenance of testicular temperature is essential for normal steroidogenesis and spermatogenesis. Results of the current study revealed a temperature-dependent difference in CRH dose-responsiveness of TM3 cells, a testicular Leydig cell line. This difference was not restricted to the Leydig cell line since AtT20 cells, a pituitary cell line, showed similar behavior. These results suggest that CRH receptors or post-receptor mechanisms may be sensitive to small changes in temperature. In the mature rat, FSH and hCG receptor number, and testosterone production decrease after cryptorchidectomy (Huhtaniemi et al., 1984; Sirvent et al., 1989). Namiki and coworkers (Namiki et al., 1987) investigated the influence of temperature on the functions of Leydig and Sertoli cells of testes from men with varicocele (scrotal temperature is elevated). hCG and FSH receptor levels were not different from men without varicocele, nor was testosterone concentrations in the testes. They also conducted experiments on human testicular organ culture at 33°C and 37°C and found no differences from controls. These studies suggest species differences. Our observations on the sensitivity of mouse cells to CRH at different temperatures could be species related.

In our studies, the EC_{50} of CRH (1×10^{-10} M) at 34°C was within the range of the

K_d of CRH (1.3×10^{-10} M) in Leydig cells, while the EC_{50} at 37°C was 1×10^{-9} M, suggesting that 34°C may be the optimum temperature for the function of CRH in testicular cells. Whether this phenomemon occurs in intact testis is not yet known.

Using immunocytochemistry, Yoon et al (1988) found irCRH-like peptide in interstitial and germ cells, while Audhya and coworkers (Audhya et al., 1989) detected irCRH-like peptide in Leydig cells but in no other cells in the testis. The reason for this descrepancy is not clear but could be due to differences in the CRH antibodies used.

E. Localization of CRH mRNA in the rat testis

We determined the possible source of CRH peptide in the testis by using a non-radioactive *in situ* hybridization technique to localize CRH mRNA. Audhya and coworkers (1987), Yoon and coworkers (1988), and Fabbri and coworkers (1990) documented the presence of immunoreactive CRH-like peptide in Leydig cells. We predicted that interstitial cells would be the predominant source of CRH mRNA. To our surprise, the digoxigenin-labelled CRH riboprobe in our studies intensely stained spermatogonia, spermatocytes, and spermatids in the tubules. Interstitial cells were not a prominantly stained cell population. These results suggest that the primary site of CRH mRNA is germ cells. The irCRH detected in Fabbri and coworkers' preparations (1990) is approximately 0.24 ng/testis, assuming that there are approximately 10 million Leydig cells/testis. This value is approximately 300-fold lower than the amount of extractable CRH-like peptide in whole rat testis reported

by Yoon *et al.* (1988). These documentations suggest that non-Leydig cells are the major source of CRH. It is possible that a Leydig cell population contains low abundance CRH mRNA level that encodes for a CRH-like peptide in low amounts; therefore, our digoxigenin detection method was not sensitive enough to detect it.

Since CRH mRNA was found in germ cells and Yoon and coworkers (1988) documented the presence of irCRH-like peptide in these cells, it is conceivable that a CRH-like peptide is synthesized in germ cells. We speculate that this CRH-like peptide may affect POMC system in spermatocytes and spermatogonia, in an autocrine/paracrine fashion since these germ cells have the capacity to synthesize POMC (Cheng et al., 1985, Gizang-Ginsberg et al., 1985). Moreover, whether a CRH-like peptide whose origin is from germ cells , could be transported into the interstitium through some mechanism, is not known. Few peptides such as LHRH-like peptide and inhibin, presumed to be synthesized in tubular Sertoli cells, have been shown to act on interstitial Leydig cells to stimulate testosterone secretion (Sharpe et al., 1981; Bardin et al., 1990). These documentations do not address the question of how these peptides could be transported from the tubules into the interstitium. Since these peptides are hydrophylic, they cannot passively transverse membranes. Active transport mechanisms between testicular tubules and interstitium have not been studied to date.

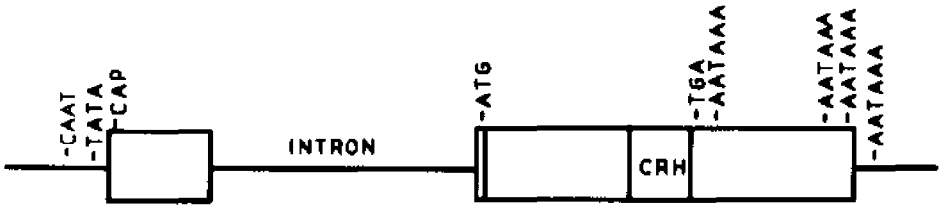
It is well established that the major function of Leydig cells is to synthesize and secrete testosterone, the necessary steroid for normal spermatogenesis and fertility.

Ulisse and coworkers demonstrated that CRH alone had no effect on testosterone secretion but that it inhibited LH-stimulated testosterone release from cultured Leydig cells (Ulisse et al., 1989). Unpublished data in our laboratory on the effect of CRH on basal testosterone production in culture confirms this finding. Some peptides have been found to stimulate steroidogenesis, such as vasopressin (Kasson et al., 1986), inhibin and interleukin-1 (Verhoeven et al., 1988; Franchimont et al., 1989), while other peptides such as activin (Bardin et al., 1990), interleukin-2 (Guo et al., 1990) and epidermal growth factor (Bellve et al., 1989), inhibit steroidegonesis in culture systems. Therefore, the action and interaction of several locally produced peptides may be important modulators of testosterone secretion.

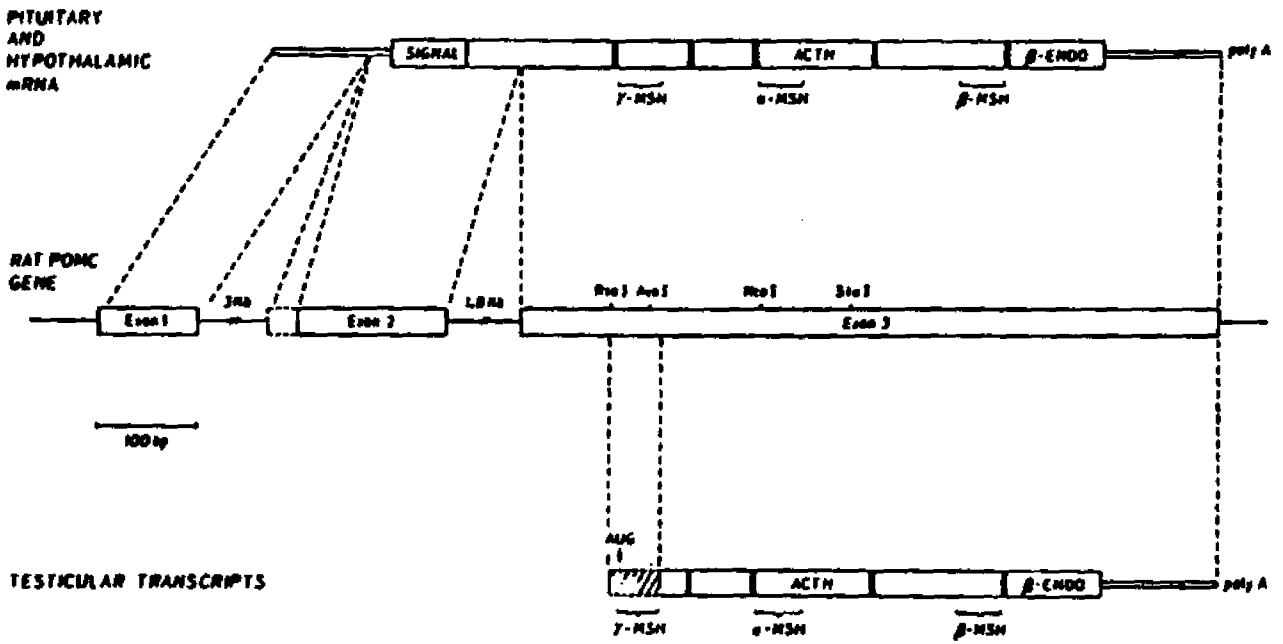
In conclusion, we clearly established that CRH is a local direct regulator of βEP secretion in the pubertal rat testis. The action of CRH is under developmental and, at least in culture, under thermal control. The factors contributing to a possible CRH short-loop regulation of testicular function are not known.

VII. APPENDIX

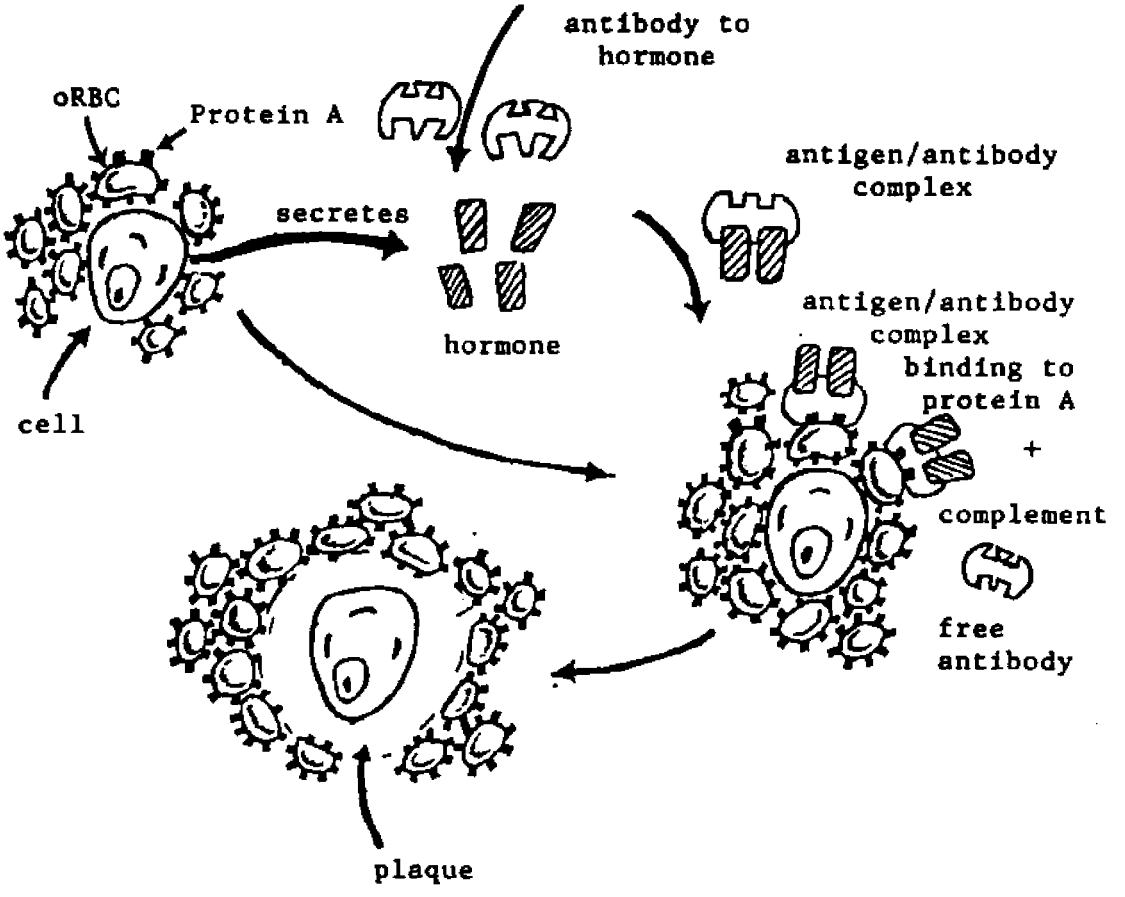
CRH GENE



POMC GENE


PITUITARY
AND
HYPOTHALAMIC
mRNA
SIGNAL
ACTH
β-END
poly A
γ-MSH
α-MSH
β-MSH
RAT POMC
GENE
3kb
LBkb
Exon 1
Exon 2
Exon 3
100 bp
AUG
TESTICULAR TRANSCRIPTS

RHPA



Drawn by Wing Chiu

RHPA is complement mediated lysis of proteinated ovine red blood cells (oRBC) in the presence of antibody-antigen complex.

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